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MASTER TESIS

Water stress impact on competition between *Tuber melanosporum* and *Tuber aestivum* on inoculated *Quercus ilex* seedlings

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1. INTRODUCTION

1.1 Mycorrhiza: A Plant-fungus-soil association

Mycorrhiza is a symbiotic structure between a photosynthetic organism and a filamentous fungus. In simple terms, it is the plant's root tip that results from being covered (ectomycorrhiza), penetrated (endomycorrhiza) or both (ectendomycorrhiza) by mycorrhizal fungi. Mycorrhizal associations exist since the life of terrestrial plants, more than 400 million years ago (Honrubia, 2009).

Most terrestrial plants live in mycorrhizal symbiosis and probably with several soil fungi. Likewise, these relationships are also established anthropically for agricultural and forestry purposes. There are seven types of mycorrhizal associations depending on the fungus, host plant and their morphologies (Fortin, Plenchette & Piché, 2015). Within the group of endomicorrhizal associations, they differs from: (1) arbusculars (*Fragaria x ananasa* with *Glomus intraradices*); (2) ericoids (*Vaccinium corymbosum* with *Rhizoscyphus ericae*); (3) orchids (*Vanilla spp* with *Ceratobasidium spp*); and (4) sebacinoids (*Zea mays* with *Piriformospora indica*). Out of the previous group, are (5) ectomycorrhizal (*Quercus ilex* with *Tuber melanosporum*); and (6) ectendomycorrhizal associations (*Helianthemum almeriense* with *Terfezia claveryi*), differing form: (7) arbutoids (*Pinus spp* with *Wilcoxina mikolae*).

Ecto-mycorrhizas are formed by the mantle and the Harting net. Both very important to explain plant benefits. The mantle is the layer of hyphae that surrounds root tips; the hyphae are fungal filaments, some of them extend through the soil. Together they increase the soil volume that the plant explores, and therefore, its absorption of water and minerals, especially of soil elements with low mobility, such as P, Zn, and Cu (Smith & Read, 2010; Ortas, 2003). The mantle also brings protection to root tips from pathogens, insects, and nematodes; and tolerance to water stress, salinity, acid soils, and heavy metals. The mycorrhizal fungi also capture and transfer organic nitrogen. In return, they receive photosynthates from the host plant. This exchange of nutrients for photosynthates occurs in the Harting Network, which is formed by hyphae.

In result, nutritional and biological benefits, decrease plant losses on the establishment and depending on the crop, they can increase and improve production and quality. In the case of truffle farming, mycorrhizal associations are obligatory for truffle production.

1.2 Mycorrhizae associations on Mediterranean Black Truffle production

Many studies demonstrate the capacity of *Quercus*, especially of *Q. ilex*, to persist in changing climates and land uses. The ability to be a thermophilic and light-demanding species with high-demand of solar radiation has favored its regeneration in extreme conditions (Castro-Díez &

Navarro, 2007; Mayoral, Calama, Sánchez-González, & Pardos, 2015; Salomón, Limousin, Ourcival, Rodríguez-Calcerrada, & Steppe, 2017; Vilagrosa, Bellot, Vallejo, & Gil-Pelegrín, 2003).

Apart from *Quercus*, the genera *Corylus*, *Castanea*, *Pinus*, *Populus*, Salix, Fagus, *Cedrus*, *Tilia*, *Betula*, *Ostrya*, and *Cistus*, have species well established and adapted to the Mediterranean basin that have associated with fungi to grow in adverse conditions. Their interest is also that they have been found forming ectomycorrhizae with *T. melanosporum* in the wild or nurseries (Morcillo, Sánchez & Vilanova, 2015).

T. melanosporum is a native and economically-interesting fungus in the Mediterranean area because its fruitbody, the Black Truffle, is a culinary delicacy well known for gourmet cuisine. Within the group of black truffles, *Tuber melanosporum* is one of the most appreciated, above of *Tuber brumale* and *Tuber indicum.* It is characterized by having a warty and black surface (peridium) and by being dark with white veins inside (glebe). The harvest in Spain takes place from the 15th of November until the 15th of March.

Despite nowadays we do not know the mechanisms that lead to truffle's production, it is known that more *T. melanosporum* ectomycorrhizae and mycelium increase the probability to form them. The fungus only produces truffles when it is well-associated with a host plant. In the forest, the most productive wild trees are those over 20 years old (observed by collectors of wild truffle). For this reason, we use mycorrhized seedlings with *T. melanosporum* for establishing our truffle orchard, reducing the waiting time. On truffle orchards, the first truffles can be collected 6-10 years after plantation. Currently, it is not difficult to obtain mycorrhized plants from specialized nurseries, but there is no standardized legislation yet for the supervision and certification of their qualities. It is one of the purposes of the sector for the next years.

1.3 Climate change on fungal competition between *T. aestivum* and *T. melanosporum*

Global wild truffle production has decreased in the last sixty years. Climate change as global warming and unstable precipitation jointly with the abandonment of the rural world, increase of forest canopy and boar population, and non-technical overexploitation are leading causes (Baragatti et al., 2019; Le Tacon et al., 2014; Reyna & Garcia-barreda, 2009). On the 1960s, most of the world wild black truffle was from France, collecting around 100 tons per year. Since the 2000 year, French production is between 15 and 30 tons per year, including the entrance of truffles from the truffle orchards (Le Tacon et al., 2014; Reyna & Garcia-barreda, 2009). Nowadays the total Spanish black truffle production is to 20 to 45 tones according to the European Group for Truffles (GET), and Federación Española de Asociaciones de Truficultores (FETT).

On unstable and unknown environments, the irrigation of truffle orchards is essential for truffle development and profitability. There is not enough experience in water management for truffle farming, a new sector that depends on fungi and its production it is underground. On the one hand, drought periods in spring decreases truffle formation. On the other hand, over 100mm per month in summer reduces mycorrhizal colonization by *T. melanosporum* replacing it by Non-*Tuber* fungi.

In 1979 Giraud describes *T. aestivum* as a *T. melanosporum* competitor. *T. aestivum* is an ectomycorrhizal fungus that in association produces the summer truffle. It has more flexible soil requirements than *T. melanosporum*, present in more clayey and silty soils, more nitrogenous, not necessarily calcareous, with over 8% of organic matter, until slightly acid soils and at altitudes since sea level (Chevalier, 2012; De Miguel, Águeda, Sánchez, & Parladé, 2014; Stobbe et al., 2013; Sánchez et al., 2016). In warm *T. melanosporum* areas in Spain without water support, *T. aestivum* is an alternative of *T. melanosporum* orchards (Sánchez et al., 2016), although *T. aestivum* is also native to areas with heavy spring and fall rain in France and Italy. According to a recent study (Garcia-Barreda, Sánchez, Marco, & Serrano-Notivoli, 2019) the range of annual precipitation of *T. melanosporum* areas in Spain is wider with a drier limit to those cited for wild *T. melanosporum* areas in France and Italy (Pacioni, 1987; Ricard et al, 2003; García-Barreda et al.,2007). So colonization of *T. aestivum* in *T. melanosporum* dry areas could be explained by the fact that *T. aestivum* is also well adapted to dry and warm climates (Pruett, 2008; Sánchez et al., 2016; Turgeman et al., 2012). Other study contrasted that *T. aestivum* was less dependent on rainfall in May in comparison to *T. melanosporum,* and that *T. aestivum* growth is not favored with an excess of water in June (Molinier et al., 2013).

In the Upper Galilee (Israel), accidentally introduced *T. aestivum* almost totally displace introduced *T. melanosporum* mycorrhizae, in just ten years, fruiting only summer truffles. There, native oak species were unexpectedly found to be better hosts for *T. aestivum* than *T. melanosporum*. The environmental conditions on the *T. melanosporum* plantation established in the Upper Galilee were better for *T. aestivum* development with more soil-climate elasticity than *T. melanosporum* (Turgeman et al., 2012). The dominance of *T. aestivum* in front of *T. melanosporum* was also recorded in a 30-year field monitoring study (Molinier et al., 2013).

In the warmest and driest *T. melanosporum* natural areas such as the Spanish Mediterranean coast and the southernmost areas (Garcia-Barreda et al., 2019), climate change could cause: directly displacement of *T. melanosporum* by colonization of other ectomycorrhizal fungi such as *T. aestivum* (Sánchez et al., 2016; Tegel, 2011); and indirectly displacement by *Non-Tuber* fungi for the necessity of complex irrigation management without experience. So in long-term conditions, knowledge of irrigation on fungal dynamics, it is a key issue for *T. melanosporum* development and truffle success.

In practical purposes, our study researches about *T. melanosporum* and *T. aestivum* mycorrhizae formation and proliferation on *Q. ilex* seedlings in different water stress regimes, and in independent and shared conditions with both fungi. We want to find out if they have the same water requirements for forming mycorrhizae or through irrigation management we can favor just *T. melanosporum*.

2. OBJECTIVES

This study aims to learn about *T. melanosporum* and *T. aestivum* differences based on our hypothesis that water potential affects their mycorrhizae formation and proliferation. At the end of the study, we want to know what happens in shared conditions: first, with mycorrhizae proliferation of each fungus on independently inoculated seedlings; and, second, with fungi ability to form mycorrhizae on non-inoculated seedlings.

We defined as dependent variable the number mycorrhizae, and as independent variables: the water stress regimes (in water potential terms), competition between the plants and the fungi.

<u>Specific objectives by inoculated and non-inoculated seedlings</u>

Determine the effect of water stress on the following parameters of inoculated seedlings (donor plants of mycorrhizae), suitable for establishing a truffle orchard:

- Plant growth in stem height and diameter and number of root tips.
- Number of mycorrhizae of *T. aestivum* and *T. melanosporum* seedlings.
- Plant competition (one inoculated with *T. aestivum* and another one with *T. melanosporum*) on plant growth and mycorrhizae proliferation.
- Fungal competition between *T. aestivum* and *T. melanosporum* for forming mycorrhizae in the same plant.

Determine the effect of water stress on the following parameters of non-inoculated seedlings (receiver plants of mycorrhizae), without mycorrhizae but suitable for fungi inoculation:

- Plant growth in stem height and diameter and number of root tips.
- Quantity of seedlings with presence of mycorrhizae.
- Number of *T. aestivum* and *T. melanosporum* mycorrhizae.
- Plant competition on plant growth.
- Fungal competition.
- Plant quality for truffle farming

3. MATERIALS AND METHODS

3.1 Establishment of the experiment

A greenhouse pot trial study was conducted from May of 2018 until February of 2019. Pots were randomly placed on the greenhouse bench without separation.

Our study was inside a plastic mesh tunnel at the School of Agrifood and Forestry Science and Engineering of the University of Lleida which is in Lleida, county Catalonia (Spain).

3.1.1 Plant material

As donor mycorrhizae plants, we purchased from a commercial nursery, one-year-old *Q. ilex* inoculated seedlings, each one containerized in 7 x 7 x 18 cm. Seedlings were independently inoculated with *T. melanosporum* and *T. aestivum* fungus. From the same nursery, as mycorrhizae receiver plants, we acquired non-inoculated pre-sprouted acorns 1-2 months old. Acorns were germinated on perlite.

Prior to planting, mycorrhizal status and plant quality were assessed. We randomly selected and examined a sample of 12 inoculated seedlings with *T. aestivum* and 12 with *T. melanosporum*, according to the methodology described by Fischer and Colinas (1996, 2014 review). We also observe microscopically, a sample of non-inoculated seedlings confirming that they did not possess any mycorrhiza before their establishment on the experiment.

In May we transplanted a total of 54 inoculated seedlings of each *Tuber* plus 144 pre-sprouted acorns, distributed in 72 plastic pots 22 cm in diameter and 19 cm in depth.

3.1.2 Substrate

Each pot was fitted with 1,680 grams (2 liters) of the same substrate as contained the inoculated seedlings, to maintain a homogenous growing medium inside it. It was composed by the plant nursery and consisted of a mixture of 15% peat, 15% coconut fiber, 15% perlite, 15% vermiculite, 20% truffle soil, 10% sand, 5% calcium carbonate, 5% earthworm humus and 180g of release fertilizer [NPK (MgO): 15-7-15 (2)]. This kind of substrate is used on truffle farming to improve soil oxygenation and humidity.

To avoid substrate losses by the impact of the drop of water, we placed on the top of each pot 3 cm depth of white crushed marble. Stones size was to 7-13 mm. This layer also avoided direct contact of the substrate with solar radiation, maintaining its humidity longer.

3.1.3 Sensors

We install a total of four MPS-6 water potential soil sensors (Meter group, Washington, USA) at a depth of 10 cm. The interest of MPS-6 soil sensors is that they cover a wide range of stress, up to -100,000 KPa. MPS-6 also measure soil temperatures. We use a Decagon Em50 data logger (Meter group, Washington, USA) for saving the MPS-6 readings. Inside the greenhouse, we install one Arduino sensor (Transfer Multisort Elecktronik, Madrid, Spain) to record air temperature.

3.2 Experimental design

We used a 6 x 2 x 3 factorial experimental design. It consisted of 6 levels of water stress (1E, 2S, 3M, 4N, 5L, 6F); 2 levels of plant competition (YES and NO) and 3 levels of fungal competition (MEL, AEST, BOTH).

One pot was the experimental unit where each treatment was applied. In competition conditions, a pot had one inoculated seedling with *T. aestivum*, one with *T. melanosporum*, and two non-inoculated. In non-competition conditions, had just one inoculated and two non-inoculated seedlings (Figure 1). Inoculated seedlings were donors of mycorrhizae, and non-inoculated were receivers of mycorrhizae.

Plant competition: No Fungal competition: AEST	Plant competition: No Fungal competition: MEL	Plant competition: Yes Fungal competition: BOTH

Figure 1. Images of types of experimental unit.

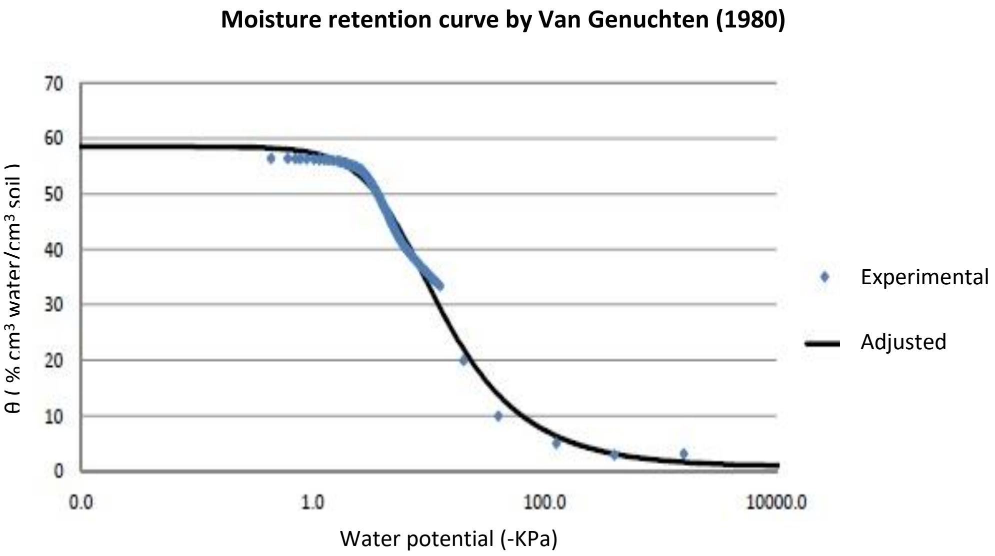
There were three repetitions, each one with a group of twenty four pots. In total six groups of twelve pots (three MEL, three AEST, six BOTH) were watered differently under greenhouse conditions from May 2018 to the end of January 2019.

3.2.1 Water stress regimes

Six regimens of water stress were chosen in terms of water potential: two where *T. melanosporum* is able to form mycorrhizae from -100 to -300 Kilopascals (KPa) and from -300 to -1,500KPa (Olivera et al., 2006); two developed on extreme conditions that Mediterranean forest species face often, over -1,500KPa and over -3,000KPa; and two, with high moisture where we would expect colonization of root tips by *Non-Tuber* fungi, from 0 to -33KPa and from -33 to -100KPa.

In order from the driest to the wettest water stress regimes with their water potential intervals were: 1E [>3,000(-KPa)], 2S [>1,500 (-KPa)], 3M [300 – 1500 (-KPa)], 4N [100 – 300 (-KPa)], 5L [33 – 100 (-KPa)], and 6F [0 – 33 (-KPa)].

We adjust the water stress intervals considering the volumetric content of water (θ) of the study substrate. This information was obtained with the adjusted curve of moisture retention by Van Genuchten (1980) (Labferrer laboratory, Cervera, Spain). The curve reflects the evolution of the volumetric water content and soil water potential (Figure 2).



Results of adjusted volumetric water content by water potential (black line) calculated by Van Genuchten expression

Water potential (-KPa)	θ (% cm^3 water/ cm^3 soil)	Water potential (-KPa)	θ (% cm^3 water/ cm^3 soil)
0	58.49	**100**	7.39
10	33.44	**500**	2.81
20	22.02	**1,000**	1.98
30	16.95	**1,500**	1.68
33	15.92	**3,000**	1.30
40	13.81	**5,000**	1.14
50	11.98	**10,000**	1.00

Figure 2. Moisture retention curve and volumetric water content evolution for our substrate. Source: Labferrer report

The laboratory used the Hyprop tensiometer (Meter group, Washington, USA) for water potential measurements until -90.0KPa and the Dew point Potential Meter WP4c (Meter group, Washington, USA) from -90.0KPa to –10,000.0KPa to elaborate the complete moisture curve (Figure 2). The experimental volumetric water content was obtained by weight measurements every two days during the drying.

The maximum moisture in saturation was 58.49% of cm^3 water/ cm^3 soil for the substrate of the study. After this point the volumetric water content decreases drastically to 15.92% in the agronomic reference of field capacity (-33KPa), losing 72.78% of maximum moisture. The substrate well drains the water against retaining moisture. Therefore, water doses were low but frequent.

The adjusted curve is result of Van Genuchten expression. It estimates the volumetric content in function of water pressure in terms of centimeters of water $\theta(h)$. Following parameters were determined by Labferrer (Figure 3).

$$\boldsymbol{\theta(h) = \theta_r + \frac{\theta_s - \theta_r}{[1 + (\alpha h)]^{1-1/n}}}$$

	Substrate results for a density of 0.84 g/cm^3 and a porosity of 69%
$h(cm\ of\ water)$: pressure in cm of water	Reading as substrate dries
$\theta_s(\ cm^3/cm^3)$: saturation volumetric water content	$\theta_s(\ cm^3/cm^3)$ = 0.585
$\theta_r(\ cm^3/cm^3)$: residual volumetric water content	$\theta_r(\ cm^3/cm^3)$ = 0.008
α and n are parameters of Van Genuchten curve $\alpha\ (1/cm)$: turbidity, water velocity through the soil $n(-)$: slope of the curve	 $\alpha\ (1/cm)$ = 0.0179 $n(-)$ = 1.752

Figure 3. Van Genuchten expression and results for the substrate.
Source: Labferrer report

Moisture curve of the substrate has a pronounced slope (n). When slope increases soil retention decreases, so clay soils have a less pronounced slope in comparison to the substrate and sandy soils similar or more pronounced it. The Residual moisture was 0.8%, content of water after dry the soil. The inverse of α indicates in which water potential starts to enter air into the soil pores for evacuating the water (Q_e). We calculate it with the following conversion.

$$Q_e(-KPa) = \frac{1}{\alpha\left(\frac{1}{cm}\right)} x \frac{1HPa}{1\ cm} x \frac{0.1KPa}{1HPa};\ Q_e(\alpha = 0.0179) = 5.59$$

(Expression 1)

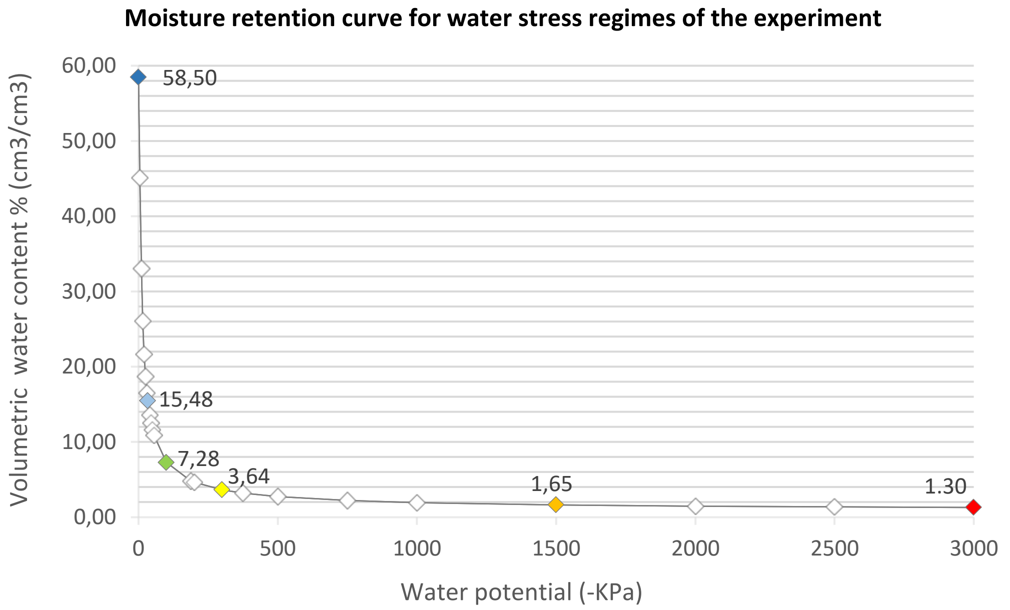
The -5.59KPa water potential corresponds to a volumetric water content of 41.29%. So it is not possible to define a water potential from 0KPa to wetter than -5.59KPa because it will produce hypoxia conditions without oxygen.

Despite water potential values are independent of soil characteristics, their volumetric water contents are not (Porta, López-Acevedo & Poch, 2009). The same volumetric content is retained with higher energy when the soil is drying than when it is moistening (Porta, López-Acevedo

& Poch, 2009). In the Labferrer laboratory, the measurements to build the curve of moisture retention were start from a saturated substrate to a dried substrate.

We consider soil sensors precision for deciding water stress regimes apart of bibliography references and evolution of water through our substrate. In our case, we used water potential sensors with an accuracy of ± 10% of readings in –KPa whose not limit our intervals.

The objective intervals of water stress were defined in terms of water potential and volumetric water content. They are represented by the moisture retention curve (Figure 4).



Water potential and associated volumetric water content by water stress regimes

Water stress regimes	Group description	Water potential interval (-KPa)	θ (% cm^3 water/ cm^3 soil)
1E	EXTREME	>3,000	≤1.30
2S	SEVERE	>1,500	1.65 – 1.30
3M	MODERATE	300 – 1,500	3.64 – 1.65
4N	NONE	100 – 300	7.28 – 3.64
5L	LUXURY	33 – 100	15.48 – 7.28
6F	FIELD CAPACITY	0 – 33KPa	58.50 – 15.48

Figure 4. Moisture retention curve for water stress regimes.

3.2.2 Plant competition

Competition between plants reflects the possible effects of having two inoculated seedlings in the experimental pot instead of one, on the individual growth of plants (stem height, diameter, number of root tips), and mycorrhizae formation. It is a direct effect for inoculated seedlings and indirect for non-inoculated.

For our experimental design, plant competition variable has two levels: YES, formed by one inoculated seedling with *T. aestivum* and another with *T. melanosporum*; and NO, when there is just one inoculated seedling with one of the fungi.

3.2.3 Fungal competition

It is the competition between fungi to form mycorrhizae in non-inoculated seedlings. In the case of inoculated seedlings, reflect the possibility of finding *T. melanosporum* mycorrhizae on initial *T. aestivum* seedlings and vice versa.

For our experimental design, fungal competition variable has three levels: AEST, with one seedling inoculated with *T. aestivum*; MEL, with one seedling inoculated with *T. melanosporum*; and BOTH, with one seedling with *T. aestivum* and another with *T. melanosporum*.

3.3 Management of the experiment: estimated water for irrigation

The amount of water needed to maintain the water potential within the objective range was calculated from the continuous readings of the sensors in the treatments 3M, 4N, 5L, and 6F. In treatment 1E and 2S we estimated the water irrigation and frequency in the first weeks of the study from the MP6 sensor readings and applied that amount throughout the experiment (Table 1). Water irrigation was the difference between the water inside the pot and the water objective, following the next steps:

1) **Reading of Water potential at pre-dawn and midday (-KPa).**
2) **Transformation from water potential to volumetric content by Van Genuchten.**
3) **Estimation of water per pot knowing that each one is filled with 2000 cm^3 of substrate.**

$$\text{Water inside the pot } (\text{cm}^3) = \theta\left(\frac{\text{cm}^3\text{ water}}{\text{cm}^3\text{ soil}}\right) \text{x } 2000\text{ cm}^3\text{ substrate}$$

θ : Volumetric water content: Vol

(Expression 2)

4) **Direct transformation from water potential to water inside a pot by the next polynomial expression.**

$y = 0.0597x^3 - 0.3464x^2 + 0.0002x + 3.085$ $R^2 = 0.9996$	$x = \log\ (-KPa)$ $y = \log\ (cm^3)$

(Expression 3)

It has a limitation for the wettest situation 0 (-KPa) that is corrected changing it for 0.65 (-KPa).

5) **Dose of water for each pot, as the difference between objective water potential and reading.**

   For example, in the 4N regime, a reading of 300 (-KPa) has an objective of 100 (-KPa), equal to 73.68 cm^3 and 150.45cm^3 water inside the pot, respectively. So the dose of water to move to the objective situation is 150.45 —73.68 = 76.77cm^3 ~ 80ml. The water was applied manually to the central area of the pot with a measuring cylinder.

Table 1. Water irrigation and estimated frequency for water stress regimes

Water stress regimes	Water potential reading (-KPa)	Objective water potential (-KPa)	Water dose per pot (ml)	Frequency (days)
1E	-	>3,000	5	21 – 25
2S	-	>1,500	10	21 – 25
3M	1,500	300	50	13 – 20
4N	300	100	80	8 – 12
5L	100	33	170	5 – 7
6F	33	0	970	2 – 4

The table shows the maximum amount of water needed to increase the water potential from the driest to the wettest objective. Frequency of applied water is indicative since it depends on readings, except for 1E and 2S where we did not use sensors.

3.4 Data collection and statistical analysis

At the end of the study, February of 2019, all the plants were removed from their pots for recording the effects of treatments. All statistical calculations were performed in R software (R Development Core Team, 2018).

Analysis of variance was performed to determine effects of water stress regimes, and plant and fungal competition by ANOVA Statistics. Means were separated using LSD post-hoc test. The confidence intervals were independently elaborated for *T. aestivum* plants, *T. melanosporum* plants and non-inoculated plants according to a normal distribution.

Water potential and temperature

Decagon Em50 data logger saved soil water potential and soil temperature readings. It did it every hour from the third week of July to the end of January. The air temperature was recorded every thirty minutes by an Arduino shield from the second week of May to the end of August.

Water irrigation and temperature

We noted the amount of water applied of each pot treatment from the second week of May to the end of January. Also we transformed water irrigation from ml to equivalent millimeters of water (liters per square meter) according to next expression:

$$Pot\ Area\ (m^2) = \pi \cdot \left(\frac{D}{2}\right)^2 ; Water\ (mm) = Water\ (ml) \cdot \frac{1l}{1000ml} \cdot \frac{1}{Pot\ Area\ (m^2)}$$

(Expression 4)

The soil temperature was grouped in five ranges. We differed three ranges of temperature over 25°C given the fact that the effect of soil temperatures to form *T. melanosporum* and *T. aestivum* mycorrhizae it is still not well defined. Ranges were: (1) Temperatures < 0°C, (2) ≥ 0°C ≤ 25°C, (3) > 25°C ≤ 30°C, (4) > 30°C ≤ 35°C, (5) > 35°C. We presented the number of consecutive hours in a day that pots were in a given temperature range. Ranges were: (1) Temperatures < 0°C, (2) ≥ 0°C ≤ 25°C, (3) > 25°C ≤ 30°C, (4) > 30°C ≤ 35°C, (5) > 35°C. We calculated the mean and the maximum number of consecutive hours into the temperature range to detect its continual exposition. We calculated the mean and the maximum number of consecutive hours into the temperature range to detect its continual exposition. Also, we calculate the total hours into a temperature range for every month and all the experiment. Totals were not with consecutive hours, just with hours into the temperature ranges.

The air temperature was differenced by two ranges: (1) Temperatures < 34°C, and (2) Temperatures ≥ 34°C. We calculated the number of hours into a temperature range. The mean, the maximum and the minimum. The number of hours was expressed for each temperature range and month. The total number of hours was from May to August.

Plant survival rate (%)

We calculate the survival rate as the quotient between Alive plants and Total plants (Alive plus Dead). We categorized plants in Alive class those with green and hardened leaves, green shoots, and those that regrowth after defoliation; and Dead class those that had all their leaves senescent and did not regrowth after defoliation.

Plant growth and number of mycorrhizae

Plant measurements were did it before and after water stress regimes. The stem height was measured from stem collar to its dominant tip and the stem diameter since 1cm from stem collar. Plant growth was defined as the difference in stem height and stem diameter measurements between after and before treatments. In the non-inoculated seedlings, we did not measure stem diameter before planting, only after planting.

The number of root tips and mycorrhizae of the donor plants were measured before and after regimes, according to the methodology described by Fischer and Colinas (1996). The number of root tips of the receiver plants was measured just after treatments, according to the same methodology. Also, we follow their criteria to evaluate the quality of receiver plants for truffle farming. We did it for the receiver plants obtained independently mycorrhized with *T. aestivum* and *T. melanosporum*.

It was not possible to obtain information about the number of root tips and the number of mycorrhizae in the same plant before and after the regimes because their counting was do it by a destructive analysis.

Relationship between plant growth and mycorrhizae

The linear correlation between the number of mycorrhizae and growth parameters was did it by Pearson's coefficient (p). It takes absolute values from 0 (no linear correlation) to 1 (perfect correlation) and indicates the type of relationship: (+) direct; (-) indirect. Pearson's coefficient also gives information about the proportion of explained variability of the variable "y" by the variable "x" as $p^2=R^2$.

4. RESULTS

4.1 Mycorrhizal status and plant quality before planting

Mycorrhizal and growth parameters of plants are reported in Table 2.

Table 2. Mean and 95% confidence interval of mycorrhizal status and plant quality for *T. aestivum* and *T. melanosporum* donor plants

Observed parameters	*T. aestivum plants*	*T. melanosporum plants*
Stem height (cm)	23.00 (18.70 – 27.29)	15.33 (13.63 – 17.03)
Stem diameter (mm)	6.75 (6.14 – 7.36)	4.83 (4.47 – 5.20)
Number of root tips	9,856 (7,220 – 12,492)	3,581 (2,725 – 4,436)
Number of *Tuber* mycorrhizae	5,100 (3,359 – 6,842)	1,661 (1,280 – 2,041)
Number of *Non-Tuber* mycorrhizae	828 (0 – 1959)	6 (0 – 16)
Root tips colonized with *Tuber* (%)	51 (43 – 58)	47 (40 – 55)
Root tips colonized with *Non-Tuber* (%)	8 (0 – 19)	1 (0 – 2)

Percent of T. *aestivum* mycorrhizae was between 43 and 58 of total root tips (mycorrhized and non-mycorrhized) and percent of *T. melanosporum* mycorrhizae was between 40 and 55% of total root tips.

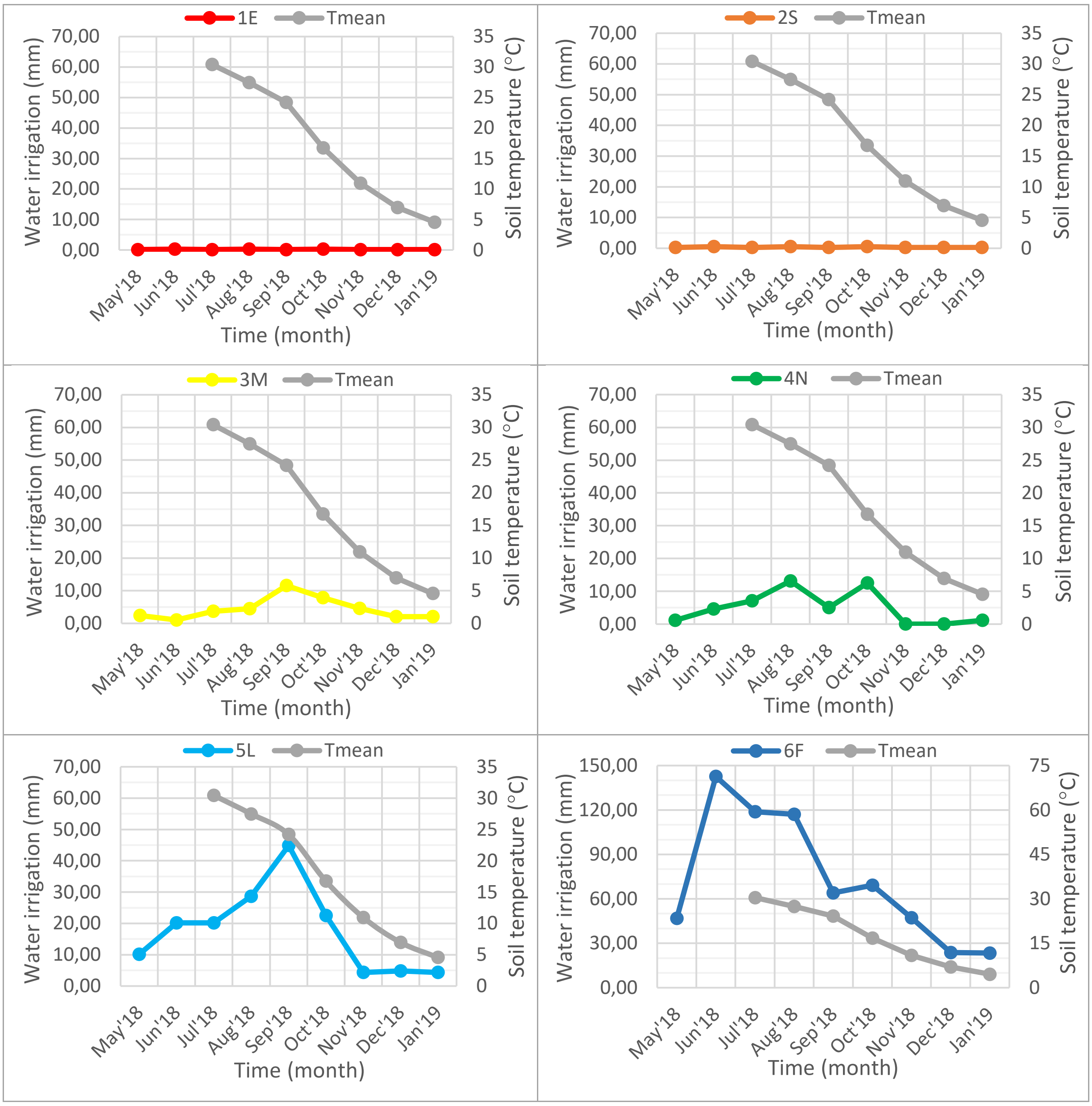
We identified in some plants mycorrhizae formed by *Sphaerosporella spp* and *Cenococumm spp*. Also, we found *dikaryotic* hyphae from a *Basidiomycota* fungus. *Non-Tuber* mycorrhizae were 8% of *Tuber* mycorrhizae in *T. aestivum* plants and 2% in *T. melanosporum* plants.

In the plant evaluation, we also confirmed that the receiver plants did not have any mycorrhiza before their establishment on the experiment.

4.2 Water irrigation and temperature

In the wettest regime (6F) we irrigated with a total of 24,810 ml which corresponds to 652.89 mm, and in the driest regime with a total of 60ml, corresponding to 1.58 mm (Figure 5). The total irrigated water on the 5L regime was 24.5% of the total irrigated on 6F. On 4L regime represented a 6.85%, on 3M a 6.11%, on 2S a 0.48% and on 1E a 0.24% of 6F.

The mean soil temperature (Tmean), minimum (Tmin) and maximum (Tmax) were presented for each month in Figure 5. The maximum temperature was 40.7 °C in August, and the minimum was -1.9°C in January.



Water irrigation in mm of water by water stress regimes and soil temperature in °C

Months	1E	2S	3M	4N	5L	6F	Tmean	Tmin	Tmax
May'18	0.13	0.26	2.37	1.18	10.13	46.84	-	-	-
Jun'18	0.26	0.53	1.05	4.61	20.13	142.63	-	-	-
Jul'18	0.13	0.26	3.68	7.11	20.13	118.82	30.4	21.6	39.2
Aug'18	0.26	0.53	4.47	13.16	28.68	117.11	27.5	15.7	40.7
Sep'18	0.13	0.26	11.58	5.00	44.87	63.95	24.2	11.7	35.6
Oct'18	0.26	0.53	7.89	12.50	22.50	69.21	16.7	2.7	28.8
Nov'18	0.13	0.26	4.61	0.00	4.34	47.24	11.0	2.3	22.5
Dec'18	0.13	0.26	2.11	0.00	4.87	23.68	7.0	1.8	14.7
Jan'19	0.13	0.26	2.11	1.18	4.34	23.42	4.6	-1.9	15.7
Total	1.58	3.16	39.87	44.74	160.00	652.89	-	-	-

Figure 5. Water irrigation for water stress regimes (mm) and soil temperature inside the pot (°C).

Table 3 collects the number of consecutive hours in a day with each soil temperature range and the total hours into the ranges. In August the temperature of the pots was >35°C during a maximum of eight hours and a mean of five hours in a day. It was the maximum consecutive hours of the experimental period that soil temperature was >35°C. Temperatures >30°C represented 53% of the total hours of the month in July and 34% in August. Temperatures dropped below 0°C only in the month of January reaching a minimum of -1.9°C.

Table 3. Number of consecutive hours into the soil temperature ranges (°C) in a day and for each month, and total hours for months and ranges

Month	Temperature range	Mean of consecutive hours in a day	Maximum of consecutive hours in a day	Total Hours
July'18	≥0 T°C ≤ 25	6	8	25
	>25 T°C ≤30	3	5	26
	>30 T°C ≤35	3	4	27
	T°C >35	6	7	30
			Total hours of July'18	**108**
August'18	≥0 T°C ≤ 25	8	13	306
	>25 T°C ≤30	2	5	182
	>30 T°C ≤35	3	7	160
	T°C >35	5	8	96
			Total hours of August'18	**744**
September'18	≥0 T°C ≤ 25	8	16	424
	>25 T°C ≤30	3	8	174
	>30 T°C ≤35	5	7	120
	T°C >35	2	2	2
			Total hours of September'18	**720**
October'18	≥0 T°C ≤ 25	17	24	699
	>25 T°C ≤30	5	7	45
			Total hours of October'18	**744**
November'18	≥0 T°C ≤ 25	24	24	720
			Total hours of November'18	**720**
December'18	≥0 T°C ≤ 25	24	24	744
			Total hours of December'18	**744**
January'19	T°C <0	7	10	74
	≥0 T°C ≤ 25	16	24	670
			Total hours of January'19	**744**
Total hours for each temperature range		**T°C <0**		**74**
		≥0 T°C ≤ 25		**3,588**
		>25 T°C ≤30		**427**
		>30 T°C ≤35		**307**
		T°C >35		**128**
			Total hours from July'18 to January'19	**4,524**

Soil temperatures ≥0 T°C ≤ 25 were the most common from the experimental period. They were 79% of total hours.

The mean air temperature (Tmean), minimum (Tmin) and maximum (Tmax) were presented in Table 4. The number of hours into the air temperature ranges is collected in the same table.

Table 4. Air temperature (°C) inside the greenhouse and number of hours into temperature ranges

Months	TMean	TMin	TMax	Hours in a month < 34°C	Hours in a month ≥ 34°C	Total Hours
May'18	21.5	9.6	37.3	240	24	**264**
Jun'18	24.6	11.9	41.0	630	90	**720**
Jul'18	27.8	15.6	42.1	559	185	**744**
Aug'18	30.3	17.4	44.8	477	267	**744**
Total hours from May'18 to Aug'18				**1906**	**566**	**2472**

August was the hottest month. In this month, there was a maximum air temperature of 44.8°C and a mean of 30.3°C, and in 56% of the hours of the month temperature was ≥34°C.

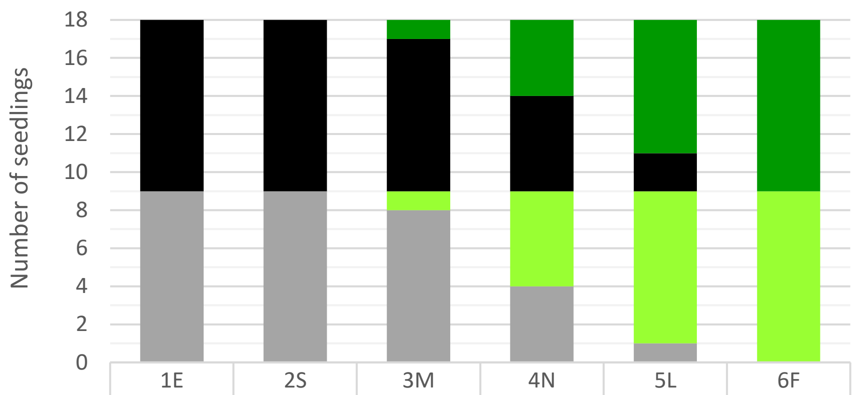
4.3 Plant growth

4.3.1 Donor plants

Survival rate

The survival rate for *T. aestivum* plants was 39% of 54 seedlings and for *T. melanosporum* plants was 43% of 54 seedlings. In both cases dead plants are mostly in the three driest water stress regimes: 1E, 2S, and 3M (Figure 6).

On 1E and 2S regimes all the plants were dead at the end of the experiment. The 6F regime was the only one that had a survival rate of 100%. Plants in 1E, 2S, and 3M water stress regimes were not further analyzed due to the low survival rate in those treatments.



	1E	2S	3M	4N	5L	6F
Alive aestivum	0	0	1	4	7	9
Dead aestivum	9	9	8	5	2	0
Alive melano	0	0	1	5	8	9
Dead melano	9	9	8	4	1	0

Results of Survival rate by water stress regimes

Water stress regimes	Water potential interval (-KPA)	*T. aestivum* plants	*T. melanosporum* plants
1E	>3,000	0%	0%
2S	>1,500	0%	0%
3M	300 – 1,500	11.10%	11.10%
4N	100 – 300	55.50%	44.40%
5L	33 – 100	88.80%	77.70%
6F	0 – 33	100.00%	100.00%

Figure 6. Survival of the inoculated seedlings for water stress regimes

Stem height and diameter

Plants in the 6F regime had significantly different stem height and stem diameter growth from plants of the other regimes (Table 5).

Table 5. Mean and 95% confidence interval of stem parameters in *T. aestivum* and *T. melanosporum* plants, before and after water stress regimes

Cond.	***T. aestivum* plants**		***T. melanosporum* plants**	
Before	**Height (cm)**	**Diameter(mm)**	**Height (cm)**	**Diameter (mm)**
Initial	22.26 (20.14 – 24.38)	6.20 (5.57 – 6.84)	11.72 (10.27 – 13.17)	3.76 (3.37 – 4.14)
After	**Height growth (cm)**	**Diameter growth (mm)**	**Height growth (cm)**	**Diameter growth (mm)**
4N	+5 (0 – 10.31) b	+0.39 (0 – 1.58) b	+7.78 (1.51 – 14.05) b	+0.72 (0.13 – 1.31) B
5L	+5.89 (0.57 – 11.20) b	+1.0 (0 – 2.20) b	+8.39 (2.12 – 14.66) b	+0.78 (0.19 – 1.37) B
6F	+24.06 (18.74 – 29.37) a	+3.44 (2.25 – 4.64) a	+24.50 (18.23 – 30.77) a	+3.33 (2.74 – 3.92) A
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05				

Root tips

There was a statistical significance in the 6F regime for *T. melanosporum* and *T. aestivum* plants (Table 6). The 6F regime is the only one that allows for the abundant formation of new root tips.

Table 6. Mean and 95% confidence interval of root tips for *T. aestivum* and *T. melanosporum* plants, before and after water stress regimes

Water stress regimes	***T. aestivum* plants**	***T. melanosporum* plants**
Before	9,856 (7,220 – 12,492)	3,581 (2,725 – 4,436)
4N	9,878 (3,143 – 16,614) b	2,273 (702 – 3,844) b
5L	9,122 (2,387 – 15,857) b	2,945 (1,374 – 4,516) b
6F	21,790 (15,055 – 28,515) a	8,822 (7,250 – 10,393) a
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05		

Effect of plant competition on growth parameters

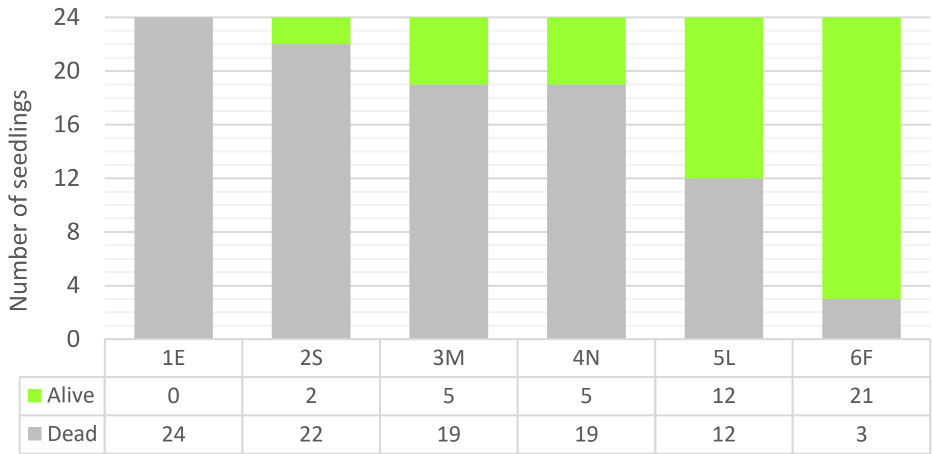
There was no statistically significant difference in stem height, stem diameter and the number of root tips between plants growing alone in a pot and plants growing with another inoculated plant next to it.

4.3.2 <u>Receiver plants</u>

Survival rate

The survival rate of the non-inoculated plants was 31% of 144 seedlings. Dead plants are mostly in the four driest water stress regimes: 1E, 2S, 3M and 4N (Figure 7).

There were plant losses in all the water stress regimes for the non-inoculated plants. The maximum survival rate was obtained in the 6F regime. Plants in 1E, 2S, and 3M water stress regimes were not further analyzed due to the low survival rate in those treatments.



Results of Survival rate per water stress regimes

Water stress regimes	Water potential Interval (-KPa)	Non-inoculated seedlings
1E	>3,000	0%
2S	>1,500	8.33%
3M	300 – 1500	20.80%
4N	100 – 300	20.80%
5L	33 – 100	50.00%
6F	0 – 33	87.50%

Figure 7. Survival of the non-inoculated seedlings for water stress regimes.

Stem height and diameter

The 6F regime was the only one significantly different than the others in stem height growth and stem diameter growth after regimes (Table 7).

Table 7. Mean and 95% confidence interval of stem parameters for non-inoculated plants, before and after water stress regimes

Water stress regimes	**Non-inoculated**	
Before	**Height (cm)**	**Diameter (mm)**
Initial	9.57 (8.07 — 11.0)	-
After	**Height growth (cm)**	**Diameter (mm)**
4N	+3.85 (1.73 — 5.97) b	2.10 (1.51 — 2.70) b
5L	+5.90 (3.78 — 8.02) b	2.44 (1.84 — 3.03) b
6F	+11.58 (9.46 — 13.70) a	3.48 (2.89 — 4.07) a
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05		

Root tips

The 6F regime was the only one with significantly higher number of root tips than the others (Table 8).

Table 8. Mean and 95% confidence interval of root tips for non-inoculated plants after water stress regimes

Water stress regimes	Non-inoculated plants
4N	21 (0 – 347) b
5L	68 (0 – 393) b
6F	1,241 (915 – 1,567) a
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05	

Effect of plant competition on growth parameters

There was no statistically significant difference in stem height, stem diameter and the number of root tips due to plant competition. The growth of the receiver plants was the same in a pot with two inoculated plants or just with one. Also, there were no differences in the survival of these plants among plant competition levels.

4.4 Mycorrhizae formation and proliferation of *T. melanosporum* and *T. aestivum*

4.4.1 <u>Donor plants</u>

Effect of water stress regimes on mycorrhizae *formation and proliferation*

We observed young *Tuber* mycorrhizae more or less developed on 5L and 6F regimes, most of them in the 6F regime. The 6F regime was the only one that maintained at the end of the experiment the amount of *Tuber* mycorrhizae (Table 9 and 10). In the rest of the regimes, the number of *Tuber* mycorrhizae decreased. *Non-Tuber* mycorrhizae were no significant between regimes.

After the water stress regimes, *T. aestivum* plants had more *Non-Tuber* mycorrhizae than *T. melanosporum* plants. The mycorrhizae of *Non-Tuber* fungi on *T. aestivum* plants were around 8% of *T. aestivum* mycorrhizae in 5L and 6F regimes. On *T. melanosporum* plants, they were less of 1% of *T. melanosporum* mycorrhizae in 4N and 6F regimes.

Table 9. Mean and 95% confidence interval of *T. aestivum* and *Non-Tuber* mycorrhizae in *T. aestivum* plants, before and after water stress regimes

Water stress regimes	***T. aestivum***	***Non-Tuber fungi***
Before	9,856 (7,220 – 12,492)	828 (0 – 1,959)
4N	3,738 (2,814 – 4,662) b	No presence
5L	4,413 (3,489 – 5,337) b	326 (300 – 352) No significance
6F	8,270 (5,554 – 10,986) a	734 (68 – 1,400) No significance
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05		

Table 10. Mean and 95% confidence interval of *T. melanosporum* and *Non-Tuber* mycorrhizae in *T. melanosporum* plants, before and after water stress regimes

Water stress regimes	***T. melanosporum***	***Non-Tuber* fungi**
Before	3,581 (2,725 – 4,436)	6 (0 – 16)
4N	745 (0 – 1,670) b	2 (0 – 28) No significance
5L	1,257 (333 – 2,181) b	No presence
6F	3,128 (2,204 – 4,052) a	20 (0 – 46) No significance
Mean comparison by LSD post-hoc test; Significance letters (a, b) for a Prob(>F)<0.05		

Effects of plant and fungal competition on mycorrhizae formation

There was no effect of plant competition on *T. aestivum* and *T. melanosporum* mycorrhizae formation. The fact of having two plants inoculated in one pot does not limit their mycorrhizae formation.

There were no observed *T. aestivum* mycorrhizae on *T. melanosporum* plants and vice versa at the end of the experiment.

4.4.2 Receiver plants

There was not presence of mycorrhizae in all the plants, there was in 37.50% of 48 plants (5L and 6F regimes). In 4L regime, there was no mycorrhizae formation. We distinguished five classes of mycorrhizae, according to their morphology and possibility of identification: (1) *T. aestivum*, (2) *T. melanosporum* (3) *Non-Tuber* fungi (*Sphaerosporella spp*, and *Cenococumm spp*), (4) Unidentified mycorrhizae and (5) Total number of mycorrhizae as the sum of all the classes. We observed a *dikaryotic* hyphae from a *Basidiomycota* fungus. Figure 8 shows photos of mycorrhizae types and fungal structures observed through a compound and a binocular microscope during the analyses.

Although *T. melanosporum* and *T. aestivum* were the two fungi to be studied, it was decided to count *Non-Tuber* mycorrhizae because they were frequent and sometimes even more than *Tuber* mycorrhizae. Five plants had presence of *T. aestivum* mycorrhizae*,* four of *T. melanosporum*, eight of *Non-Tuber* fungi and twelve of Unidentified mycorrhizae. We found more than one mycorrhizae type in a plant.

The Unidentified mycorrhizae were thin and young and had no *cystidia*. Their mantle was not clearly differentiated and did not cover the root all the way to the tip. In these cases, it was not possible to distinguish between a young mantle of *T. melanosporum* and a one of *Sphaerosphella spp* since the two have an irregular mantle that reminds of a puzzle. The *T. melanosporum* mantle is much more defined as a puzzle when the mycorrhiza is mature. Sometimes the mantle color of *Sphaersphella spp* is darker than that of *T. melanosporum*, which helps its identification. On the other hand, the distinction between the mantles of *T. aestivum* and *Sphaerosphorella spp* was clearer, being the *T. aestivum* mantle polygonal and the *Sphaerosphorella spp* a thick puzzle. Figure 8 shows photos of the comparisons and differences.

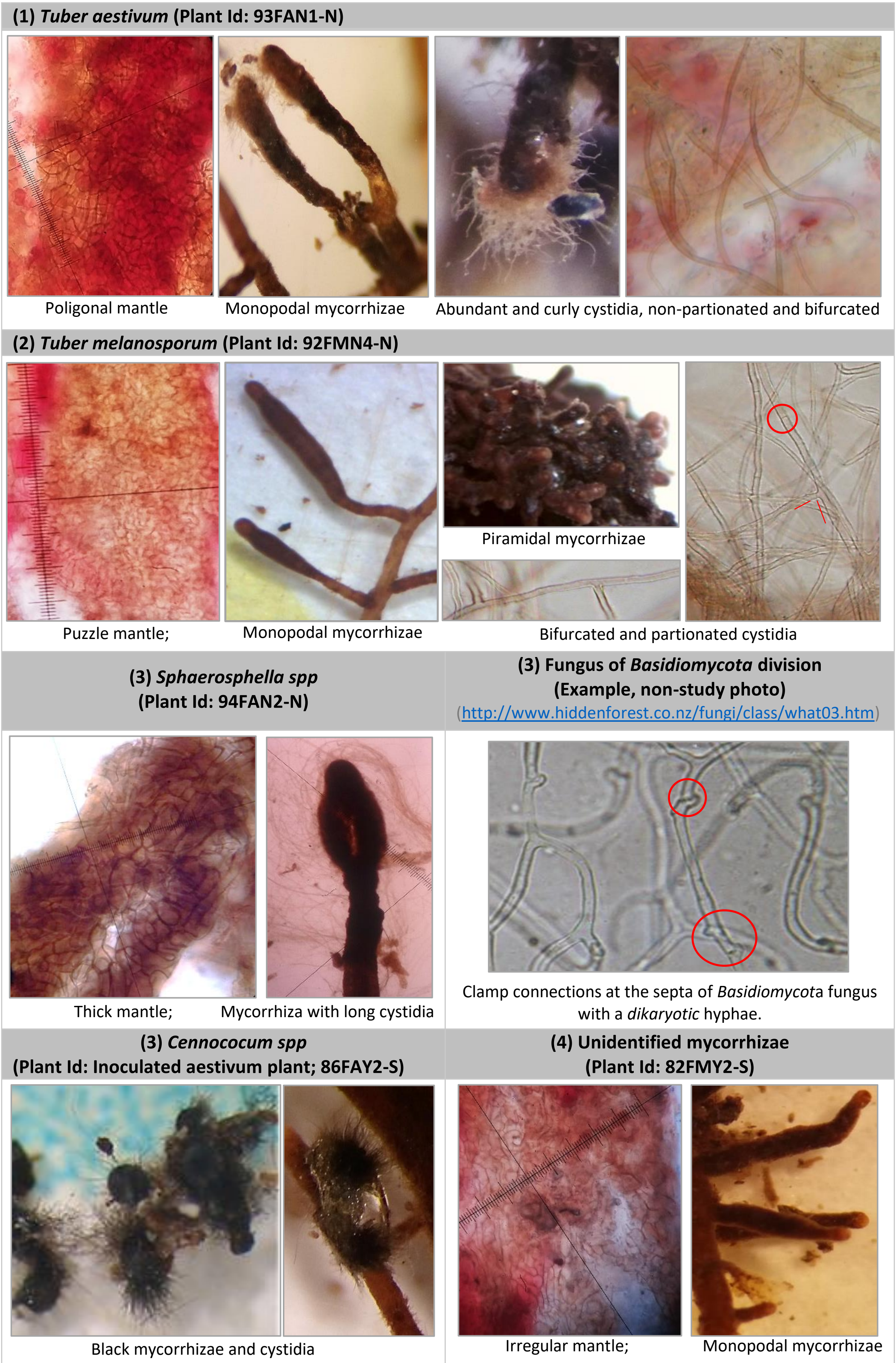
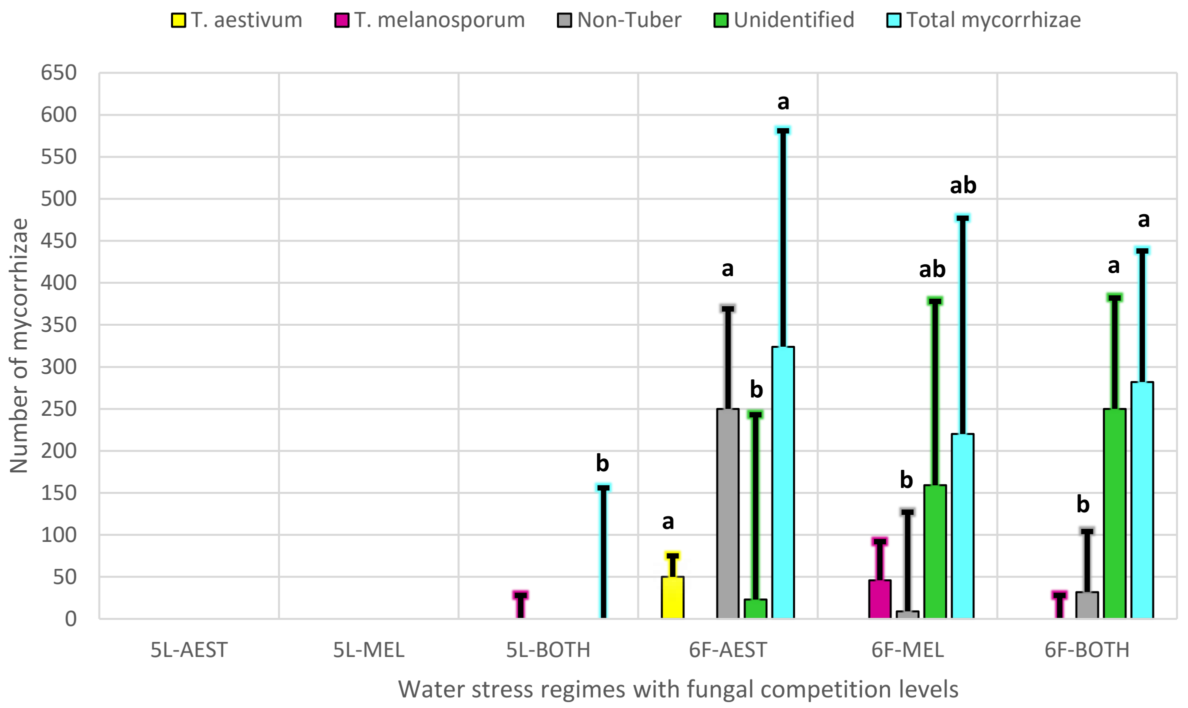


Figure 8. Optical and binocular microscope photos of the observed mycorrhizae classes on non-inoculated seedlings.

Effect of water stress regimes and fungal competition on mycorrhizae *formation*

There was an interaction between water stress regimes and fungal competition on *T. aestivum* mycorrhizae and *Non-Tuber* mycorrhizae. So we analyzed the number of mycorrhizae by water stress regimes with fungal competition levels, obtaining the groups: 5L-AEST, 5L-MEL, 5L-BOTH, 6F-AEST, 6F-MEL, 6F-BOTH (Figure 9).



Mean and 95% confidence interval of mycorrhizae for non-inoculated plants, at the end of the experiment, by water stress regimes and fungal competition conditions.

Conditions	*T. aestivum*	*T. melanosporum*	*Non-Tuber*	*Unidentified*	*Total mycorrhizae*
5L-AEST	No presence	-	No presence	No presence	No presence
5L-MEL	-	No presence	No presence	No presence	No presence
5L-BOTH	No presence	0 (0 – 28) No significance	No presence	No presence	0 (0 – 156) b
6F-AEST	50 (24 – 75) a	-	250 (132 – 369) a	23 (0 – 243) b	324 (67– 581) a
6F-MEL	-	46 (0 – 92) No significance	9 (0 – 127) b	159 (0 – 378) ab	220 (0 – 477) ab
6F-BOTH	No presence	0 (0 – 28) No significance	32 (39 – 104) b	250 (117 – 382) a	282 (126 – 438) a

Mean comparison by LSD post-hoc test; Significance letters (a, ab, b) for a Prob(>F)<0.05

Figure 9. Number of mycorrhizae for water stress regimes and fungal competition conditions.

In this study, we observed that *T. aestivum* mycorrhizae just appeared on the 6F irrigation regime and without the fungal competition of *T. melanosporum* (6F-AEST). There were no significant differences between the number of *T. melanosporum* mycorrhizae in the wettest regimes and the fungal competition. However, it formed mycorrhizae in the 5L-BOTH regime with competition, where *T. aestivum* could not. *T. melanosporum* and *T. aestivum* were not found together on any receiver plant of mycorrhizae.

The *Non-Tuber* mycorrhizae was only present on the wettest regime 6F. The mean of *Non-Tuber* mycorrhizae was significantly higher in *T. aestivum* pots than in *T. melanosporum* pots or pots with both fungi (Figure 9).

The mean of unidentified mycorrhizae was 7% of total mycorrhizae in 6F-AEST, and 70% in 6F-MEL. The amount of unidentified mycorrhizae is significantly higher on 6F-MEL and significantly lower on 6F-AEST.

4.5 Relationship between plant growth and mycorrhizae

4.5.1 Donor plants

All the relations between plant growth and the number of mycorrhizae were direct (Figure 10). Number of mycorrhizae increases with root tips, stem height and diameter growth. The p-values of these linear relationships is reflected in colors. All are above 95%.

Pearson's coefficient table

Factor y - Factor x	***T. aestivum* plants**	***T. melanosporum* plants**
Mycorrhizae - Root tips	+0.92	+0.83
Mycorrhizae - Stem height growth	+0.53	+0.81
Mycorrhizae - Stem diameter growth	+0.59	+0.75

p-value	**Color**
<0.001	
<0.01	
<0.05	
<0.1	
<1	

Figure 10. Pearson's coefficient table between mycorrhizae and growth parameters of *T. aestivum* and *T. melanosporum* donor plants. It is colored by p-value significance.

The number of root tips explained an 85% of mycorrhizae formation variability for *T. aestivum* and a 69% for *T. melanosporum*.

4.5.2 Receiver plants

All the mycorrhizae classes increases with root tips, root height, stem height growth and stem diameter (Figure 11). The correlation was more significant when it was calculated with Total mycorrhizae. The number of root tips was the plant parameter than explained more of mycorrhizae formation variability.

Pearson's coefficient table

Factor y - Factor x	PAa	PMa	PCa	PUa	PMTa
Mycorrhizae - Root tips	+0.63	+0.49	+0.52	+0.54	+0.83
Mycorrhizae - Stem height growth	+0.22	+0.15	+0.35	+0.42	+0.55
Mycorrhizae - Stem diameter	+0.21	+0.16	+0.26	+0.46	+0.54

p-value	Color
<0.001	
<0.01	
<0.05	
<0.1	
<1	

PAa: *T. aestivum* mycorrhizae; **PMa:** *T. melanosporum* mycorrhizae

PCa: *Non-Tuber* mycorrhizae; **PUa:** Unidentified mycorrhizae; **PMTa:** Total mycorrhizae

Figure 11. Pearson's coefficient table between mycorrhizae and growth parameters of receiver plants. It is colored by p-value significance.

4.6 Quality of receiver plants for truffle farming

The *T. aestivum* plants obtained in 6L-AEST conditions were limited in the number of *T. aestivum* mycorrhizae and the number of total root tips, according to truffle farming criteria (Fischer & Colinas, 1996). Also, they had *Non-Tuber* mycorrhizae over 25% of *T. aestivum* mycorrhizae (Table 11). In the case of *T. melanosporum* plants obtained in 6L-MEL conditions they were only limited by the number of *T. melanosporum* mycorrhizae (Table 12).

Table 11. Quality results of receiver plants in 6L-AEST conditions

***T. aestivum* plants**							
Plant Id	**Stem height (cm)**	**Stem diameter (mm)**	**PAa**	**PCa**	**PUa**	**PTa**	**Criteria and result for truffle farming**
93-N	23.00	5.00	46	340	0	1,325	Does not pass. PAa <33% of PTa. Also, PTA is <1800, and PCa> 25% of PAa.
93-S	17.00	5.00	0	0	14	27	Does not pass. No presence of PAa and low amount of root tips.
94-N	15.00	3.00	169	537	0	4,033	Does not pass. PAa <600, condition when PTA is high. Also PCa >25% of PAa.
94-S	20.50	4.00	46	616	0	2,088	Does not pass. PAa <33% of PTa. Also, PCa >25% of PAa.
96-N	6.00	3.00	1	0	17	232	Does not pass. No amount of PAa and PTa. Also stem height is shorten than 8cm.
96-S	9.00	4.00	37	9	110	2,180	Does not pass. PAa <33% of PTa. Also does not if PUa is all of PAa

PAa: Number of *T. aestivum* mycorrhizae; **PCa:** Number of *Non-Tuber* mycorrhizae; **PUa:** Number of unidentified mycorrhizae; **PTa:** Number of total root tips
In green the suitable parameters for truffle farming, and in red not suitable.

Table 12. Quality results of receiver plants in 6L-MEL conditions

***T. melanosporum* plants**							
Plant Id	**Stem height (cm)**	**Stem diameter (mm)**	**PMa**	**PCa**	**PUa**	**PTa**	**Criterias and result for truffle farming**
90	27	5.5	0	0	951	2,475	Does not pass. No presence of PMa. It passes if PUa was formed just by PMa. Or with some PCa < 25% of PMa).
92	17	4	276	55	0	4,398	Does not pass. PMa <600, condition when PTa is high.
PMa: Number of *T. aestivum* mycorrhizae; **PCa:** Number of *Non-Tuber* mycorrhizae; **PUa:** Number of unidentified mycorrhizae; **PTa:** Number of total root tips. In green the suitable parameters for truffle farming, and in red not suitable.							

5. DISCUSSION

5.1 Mycorrhizal status and plant quality before planting

At the beginning of the experiment, *T. aestivum* plants were much more developed than those of *T. melanosporum*. It is likely that *T. aestivum* plants were planted earlier in the nursery pots and for that reason, they presented higher values than *T. melanosporum* for all the parameters observed: stem height, stem diameter, number of root tips, number of *Tuber* mycorrhizae and number of *Non-Tuber* mycorrhizae. Donor plants were suitable for truffle farming in the number of *Tuber* mycorrhizae although there were two *T. aestivum* plants that had more than 50% of *Non-Tuber* mycorrhizae. The receiver plants did not have any mycorrhiza before they were planted in the experiment pots.

5.2 Water irrigation and temperature

We consider that the high temperature inside the greenhouse and the pots, together with a high water stress deficit could have limited the survival rate, plant growth and number of mycorrhizae in our plants.

In Toledo was conducted a field study with one-year-old *Q. ilex* seedlings where was obtained a high result of plant survival and seedling growth for a total amount of water per year and mean temperature of 460 mm and 15°C the first year, 245 mm and 16.8°C the second year, and 520 mm and 16.3°C the third year (Rey Benayas, 1998). The 95% of plants survive the first year and 85% the third year, and every year seedlings had significantly more stem diameter than those of control without support irrigation. In our study, the total water irrigated in the 6F regime was under 800 mm from the second week of May to the end of January, considered a dry climate according to the updated World map of the Köppen-Geiger (Peel et al., 2007). The other water stress regimes were under 400 mm, such as a semiarid or arid climate. In comparison to the Toledo study the 6F regime was the only one irrigated with over 520 mm and in which seedling survival was 100% of donor plants. The plants in that regime presented higher stem height, stem diameter, number of root tips and number of mycorrhizae than the others. The rest of the regimes in comparison to the Toledo study were under 245 mm in which seedling survival was around 77%, 44% and 11% of donor plants for 5L, 4N and 3M regime and was 0% for 2S and 1E regimes. Apart from the higher water stress, air and soil temperature inside the greenhouse were warmer than in field conditions. Mean air temperature was 26°C from May to August. In August air temperature was ≥ 34°C in 56% of the hours of the month and soil temperature was > 25°C in 59% of the hours of the month. It was observed that soil temperatures < 30°C favored formation of root tips and *T. melanosporum* mycorrhizae in *Q. ilex* seedlings, especially when soil water potentials are lower than -600KPa (Olivera et al 2006; Olivera, Bonet, Palacio, Liu, & Colinas, 2014), and the optimum rhizosphere temperatures for *T. melanosporum* inoculation and continued mycorrhizal development have been shown to be between 20°C and 25°C in controlled conditions (Bustan et al 2006).

In some studies it was demonstrated that occasional drought periods did not disfavor mycorrhizal colonization by *T. melanosporum* and growth of *Q. ilex* seedlings and sometimes they improved their status (Bonet, Fischer, & Colinas, 2006; Büntgen et al., 2015; Domínguez Nuñez, Planelles, Rodríguez, & Saiz de Omeñaca, 2009; Olivera et al., 2006; Olivera, Bonet, Oliach, & Colinas, 2014). However, prolonged and high water deficits accompanied by high temperatures can inhibit mycorrhizae proliferation and plant development (Olivera, Bonet, Palacio, Liu, & Colinas, 2014) as has happened in this study.

5.3 Plant growth

Dead donor and receiver plants were mostly in the three driest water stress treatments with water potentials under -300KPa. The -33KPa soil water potential was the lowest that donor plants tolerated without any plants dying, which correspond with the 6F regime. On receiver plants, there were dead plants in all the regimes.

Stem height and diameter growth, as well as root tip formation, was higher in the 6F regime than in any of the other regimes. *T. aestivum* donor plants grew the same as *T. melanosporum* plants in stem height and stem diameter although *T. aestivum* plants were initially more developed. They grew around 24 cm in stem height and 3 mm in stem diameter by the end of the experiment. On the other hand, receiver plants grew a mean of 11.58 cm in stem height and reached 3.48 mm in stem diameter by the end of the experiment. Some studies show that young seedlings inoculated with fungi such as *T. melanosporum* improve their growth and tolerate better drought than non-inoculated (Domínguez et al., 2009; Domínguez Núñez, Serrano, Barreal, & González, 2006). Apart from the effect of fungi inoculation, receiver plants were younger than donor plants. Donor plants were planted at one-year-old and receiver plants at the age of 1-2 months, which influenced their growth and survival capacity under water stress.

5.4 Mycorrhizae formation and proliferation of *T. aestivum* and *T. melanosporum*

In this study, non-inoculated seedlings were mycorrhized by *T. aestivum* and *T. melanosporum*, after being for eight months in a pot with inoculated seedlings as donor plants of mycorrhizae. *T. aestivum* and *T. melanosporum* mycorrhizae were present only in a few receiver plants and in low numbers, so it is difficult to extrapolate the results to general trends for these fungi. Also, unexpected *Non-Tuber* fungi formed mycorrhizae on the new root tips.

On receiver plants, there were mycorrhizae only in the 5L and 6F regimes, especially in the 6F that was kept between 0 and -33 KPa. On donor plants, the 6F regime was the only one that maintained at the end of the experiment the amount of mycorrhizae. The rest of the regimes had a lower number of mycorrhizae than at the beginning of the experiment. So based on our hypothesis that water potential affects mycorrhizae formation, we observed that in young seedlings and our conditions, there was no formation of *Tuber* mycorrhizae in soil water potentials lower than -100 KPa.

In a pot study, it was observed that *T. melanosporum* followed two different trends depending if it was in competition with other *Tuber* fungi or without competition. In absence of *T. brumale*, *T. melanosporum* increased under moderate irrigation (minimum of -30 KPa before irrigation) and was lower under high irrigation (to -0.1 KPa to -20 KPa). But when growing together, *T. melanosporum* prevailed on young roots in high irrigation while *T. brumale* prevailed on moderate irrigation (Mamoun & Olivier, 1993).

In our study, *T. melanosporum* formed mycorrhizae in 5L and 6F regimes without differences and regardless of the fungal competition. If there had been more receiver plants with presence of mycorrhizae of *T. melanosporum* there may have been significant differences at least between water stress regimes. Although *T. melanosporum* mycorrhizae were present only in a few receiver plants and in low numbers they were present in shared conditions and in a drier regime than *T. aestivum*. In our study, *T. aestivum* was not able to form mycorrhizae under competition conditions, and in the 5L regime although *T. aestivum* donor plants had more mycorrhizae than *T. melanosporum* plants at the beginning of the experiment. In the 6F regime, *T. aestivum* formed mycorrhizae but in similar numbers as those of *T. melanosporum*. More research is needed about mycorrhizae formation of *T. melanosporum* and *T. aestivum* under different water irrigation for corroborating if they have different trends depending on the competition situation as seen in this study and in Mamoun and Olivier (1993) or if *T. melanosporum* is always more competitive than *T. aestivum* in moister conditions as we expected.

Non-Tuber fungi were able to form mycorrhizae in the receiver plants as *Tuber* fungi did, even though they were scarce or not present in some of the donor plants. In 6L-AEST and 6L-BOTH conditions *Non-Tuber* mycorrhizae were more abundant than *Tuber* mycorrhizae. In general, *Non-Tuber* mycorrhizae were significantly more abundant in *T. aestivum* pots than in *T. melanosporum* pots or pots with both fungi, and were only present in the wettest regime. This may be related to the fact that *T. aestivum* donor plants had more *Non-Tuber* mycorrhizae before planting than *T. melanosporum* plants and that the 6F regime had an irrigation of over 100 mm per month in June, July and August, which is associated to colonization by *Non-Tuber* fungi (Mamoun & Olivier, 1989). So in wet conditions, *Non-Tuber* fungi could affect the formation of *Tuber* mycorrhizae and be dominant in plants without previous mycorrhization as happened in the receiver plants. On the contrary, the formation of *Non-Tuber* mycorrhizae in the *T. aestivum* and *T. melanosporum* donor plants were not as strong as in the receiver plants. It is possible that *Tuber* fungi were more competitive in the *T. aestivum* and *T. melanosporum* donor plants where they had high levels of mycorrhizae at the beginning of the experiment. In field conditions, *Sphaerosporella s*pp and *Cenococumm spp* fungi are not strong competitors to displace *T. melanosporum* (Reyna et al., 2004)

The Unidentified mycorrhizae of receiver plants were 7% of total mycorrhizae on 6L-AEST conditions and 70% of total mycorrhizae on 6F-MEL. It was in line with the explanation about it costs more to differentiate the young mycorrhizae of *T. melanosporum* from *Sphaerosphella spp* than of *T. aestivum* from the contaminant. In the *T. melanosporum* pots, they would have been more mycorrhizae of *Tuber* of the unidentified than *Non-Tuber* mycorrhizae of the unidentified if we assume that *T. melanosporum* inoculated plants had less contamination than *T. aestivum* plants.

In general, we observed that in our conditions, *T. aestivum*, *T. melanosporum* and *Non-Tuber* fungi could have overlapping water necessities to form mycorrhizae. So if we want to reduce *Non-Tuber* or *T. aestivum* mycorrhizae in nursery conditions, we may also lose *T. melanosporum* mycorrhizae. We cannot assure that there are no differences between *T. aestivum* and *T. melanosporum* in their conditions to form mycorrhizae because we were limited by the low number of plants with *Tuber* mycorrhizae. Further research is needed to detect the specific needs of these fungi and apply them in truffle farming.

It would be interesting for new purposes to study the formation of mycorrhizae in wet conditions for plant production, and combine soil water potential with leaf water potential or hydraulic conductivity to monitor plant status during the experiment. For example, for the three next intervals of water potential: (1) to 0 to -20 KPa, (2) to -20 to -40 KPa and (3) to -40 to -100 KPa. It could also be examined if there are overlapping water necessities to form *Tuber* and *Non-Tuber* mycorrhizae and if we must lose potential *Tuber* mycorrhizae to avoid the formation of *Non-Tuber* mycorrhizae or contrary, we can manage it through irrigation. These suggestions could be carried out infecting a substrate with spores of *T. melanosporum*, *T. aestivum* and *Non-Tuber* before planting non-inoculated seedlings.

5.5 Relationship between plant growth and mycorrhizae

The number of mycorrhizae increased with root tips, stem height and stem diameter. This correlation could not be detected when mycorrhizae were present only in a few seedlings and in low numbers, as was the case in receiver plants when we used only the data of mycorrhizae only with *T. melanosporum*, *T. aestivum*, or *Non-Tuber* mycorrhizae. We could detect correlations when we pooled all the mycorrhizae together.

The number of root tips was the parameter that best explained the number of mycorrhizae for all the plants and types of mycorrhizae.

We observed that in young seedlings, the number of mycorrhizae increases with all the growth parameters. But as the tree grows, it is possible that this relationship decreases. In a truffle orchard, the biggest trees are not necessarily the most productive. It is important to consider root renewal because the symbiosis is produced in the fine roots.

5.6 Quality of receiver plants for truffle farming

None of the receiver plants were acceptable to establish a *T. aestivum* or a *T. melanosporum* orchard according to the methodology used (Fischer & Colinas, 1996).They did not meet all the quality criteria. Most of the plants had too few mycorrhizae and root tips but they all reached an acceptable stem height and stem diameter. Also, plants in *T. aestivum* pots had too high amounts of *Non-Tuber* mycorrhizae.

These results are not as good as obtained by other inoculation systems which put in direct contact fungi spores and root tips. On the one hand, the formation of *Tuber* mycorrhizae by the indirect contact of inoculated plants was a slow process. It is not recommendable for plant production purposes. On the other hand, we were surprised by the capacity of *Non-Tuber* fungi to form mycorrhizae in the same conditions.

6. CONCLUSIONS

In our study, there was no formation of mycorrhizae in new root tips for soil water potentials lower than -100 KPa. The mycorrhizae formation by the indirect contact of inoculated plants was a slow process for *Tuber* fungi but it was faster for the unexpected *Non-Tuber*.

T. aestivum was not able to form mycorrhizae under competition conditions and in the 5L regime, and *T. melanosporum* mycorrhizae were not significantly different between the wettest regimes and fungal competition.

The plants in the 6F where the only ones that tolerated greenhouse conditions without mortality and had significantly higher stem height, stem diameter, number of root tips and number of mycorrhizae than the others. The relationship between plant growth and the number of mycorrhizae was direct and tight, so it is important to keep inoculated plants in good status.

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ANNEXES

ECONOMIC ASSESMENT OF THE PROJECT:

T. AESTIVUM AND *T. MELANOSPORUM*

IN A *T. MELANOSPORUM* ORCHARD

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1. PROJECT: *T. MELANOSPORUM* ORCHARD

This section presents a detailed example of possible economic repercussions when *T. aestivum* and *T. melanosporum* coincide in a *T. melanosporum* orchard, given the interest of our study to learn about *T. aestivum* and *T. melanosporum* trends when they are in shared conditions.

We assumed a property of 10.3ha wherein ten hectares were planted with inoculated *Q. ilex* seedlings with *T. melanosporum*. The project life span was estimated to be 40 years. We intended to estimate the Investment, Annual Receivables, and Annual Payables to analyze the economic viability of the project. We did a sensitivity analysis with free contamination conditions by T. aestivum, and different levels of contamination, truffle production yield, and market prices.

2. INVESTEMENT

2.1 Materials and labors

In general, the materials and labors needed to establish the project were for: Land purchase and preparation, installation of the irrigation system, plantation, and truffle collection.

2.1.1 Land purchase and preparation

The analysis of soil adequacy for truffle farming (pH, texture, organic matter, hydraulic conductivity and soil nutrients), purchase of the 10.3 ha that were cropped to wheat, soil labors in depth and on the surface before planting, and the installation of the fence in the perimeter.

2.1.2 Installation of the irrigation system

Drilling of the water well, construction of the water irrigation pond and the irrigation shed, and the installation of the irrigation system with micro sprinklers.

2.1.3 Plantation

The analysis of the plants for evaluating their mycorrhizal status and plant quality, purchase of inoculated seedlings with *T. melanosporum*, and defining the plant location and for planting.

2.1.4 Truffle collection

The materials for truffle collection, such as truffle knives. The purchase of trained dogs. This investment takes place in the 6th year when the plantation starts to produce truffles.

2.2 Result of Investment

The payments of investment are decomposed by activities (Table 1). The activities were described and ranked in order of realization. The investment was 220,809.06 € in the 0 year and 4,840.00 € in the 6th. The total investment was 225,649.06€. It could mean a fixed investment of 18,415.21 €/ha for all the truffle orchards plus variable investment depending on the plantation. The necessary variable payments for 10ha of truffle production were equivalent to 4,149.60€/ha and included drilling of the water well, construction of irrigation shed equipped with one pump, one sand filter and one programmer, and the construction of an irrigation pond of 4 million of liters of capacity. The rest of the investment was included in the fixed investment.

Table 1. Investment of the *T. melanosporum* orchard

Year	Activity	Activity description	Time	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
0	Land purchase	Plantation of 10.3ha with a wheat crop. 6500€/ha	Before July	Ha of truffle production	1	6,695.00	6,695.00		66,950.00 €
0	Soil analysis	Soil parameters for truffle farming. One analysis every 2ha. 99,52 €/analysis	Before July	Direct payment per hectare					497.61 €
0	Drilling of the water well*	Perforation at 150m depth (12,000€+IVA) and 20kW pump cost and installation (4,570€/IVA). Water yield 10,000 l/h.	Before July	Direct payment for 10ha					20,267.50 €
0	Construction of an irrigation shed*	Construction and installation labors (1,900€+IVA) equipped with one 3.5kW pump (800€+IVA), one sand filter (700€+IVA) and a programmer (1,000€+IVA).	Before July	Direct payment for 10ha					5,324.00 €
0	Construction of an irrigation pond*	Capacity of 4 million of liters	Before July	Direct payment for 10ha					15,905.45 €
0	Depth soil labor	Soil ripping to remove the plough layer of the plantation: Tractor driver +150 CV Tractor + ripper.	July	Hours	2	70.00	140.00	169.40	1,694.00 €
0	Irrigation system materials	Main hose, secondary hoses, micro sprinklers, micro tube, hose joints, drains.	Before August	Direct payment per hectare			2,500.00	3,025.00	30,250.00 €
0	Installation irrigation system	Opening and closing of ditches, and montage.	August	Direct payment for 10ha					16,456.00 €
0	Fence material	Perimeter fence. 6x1.40 m mesh and 2m angle sticks.	Before September	Lineal meter of perimeter	450	5.00	2,250.00	2,722.50	27,225.00 €
0	Surface soil labor	Weed removing and soil aeration: Tractor driver +150CV Tractor + plough.	September	Hours	1	60.00	60.00	72.60	726.00 €
0	Fence installation	Place meshes and hammer angles: small digger + hammer arm and laborers	September	Direct payment per hectare			440.00	532.40	5,324.00 €
0	Mycorrhizal and plant quality analysis	Plants to analyse: 1% of total plants+2 plants; 1% of 2,800 plants+2 plants = 30 plants. Equivalent to 3 plants per hectare	September	Plant equivalence	3	45.00	135.00	163.35	1,633.50 €
0	Purchase of inoculated plants	1-year-old *Q. ilex* seedlings inoculated with *T. melanosporum* for a plantation framework of 6m x 6m	October	Number of seedlings	280	7.50	2,100.00	2,541.00	25,410.00 €

Year	Activity	Activity description	Time	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
0	Define plants location	Placing with GPS	October	Direct payment per hectare			100.00	121.00	1,210.00 €
0	Plant labor	Open holes with a hoe: various two laborers	October	Daily wage	2	80.00	160.00	193.60	1,936.00 €
					Total investment in the 0 year				**220,809.06 €**
Year	**Activity**	**Activity description**	**Time**	**Factor**	**Units/ha**	**€/unit**	**€/ha**	**€/ha+VAT**	**Total 10ha**
6	Purchase material for truffles collection	Truffle knife, kneepads and bags	Before November	Direct payment for 10ha					302.50 €
6	Purchase of truffle dog	Trained dog for truffle collection. 1 dog every 2ha. 750+IVA/dog	Before November	Direct payment for 10ha					4,537.50 €
					Total investment in the 6 year				**4,840.00 €**
								Total investment	**225,649.06 €**

***Activities not included in the fixed investment and included in the variable investment**

3. ANNUAL RECEIVABLES

In a truffle orchard, Ordinary Receivables come from selling the truffles. In contrast, Extraordinary Receivables are those that do not come from selling truffles, and are not frequent, such as the sale of holm oak firewood at the end of the project life span.

3.1 Ordinary Receivables

The Ordinary Receivables from the sale of truffles depends on truffle production yield, *T. aestivum* contamination and market price of *T. melanosporum* and *T. aestivum* truffles. We defined different levels of them to evaluate a posteriori their economic viability.

3.1.1 Truffle production yield

We consider five development phases and period intervals: plantation, unproductive, entry into production, productive potential and stability, and decrease of production. Even though in truffle farming, collect a truffle is a success, there are associated payments that need a minimum of production. In this line, we classify three levels of Truffle production according to development phases: Low, Medium and High (Table 2). Yield levels correspond for a *T. melanosporum* orchard with support irrigation.

Table 2. Mean production in truffle kilos per hectare and year for development phases

Period (Year)	Development phases	Truffle yield (kilos/ha and year)		
		Low	Medium	High
0	Plantation	0	0	0
1-5	Unproductive	0	0	0
6-14	Entry into production	5	10	20
15-30	Productive potential and stability	15	30	60
31-40	Decrease of production	7,5	15	30

We considered that production starts in the 6th year and decreases from year 30 when the management of the plantations is not intensive. There is not enough information about the productive life span of truffle orchards because it is a new farming sector in Spain that does not have too old plantations.

3.1.2 *T. aestivum* contamination

There may be several sources of *T. aestivum* contamination into a *T. melanosporum* orchard: first is the purchase of inoculated seedlings with mycorrhizae of *T. aestivum* instead of *T. melanosporum*; second the contamination of surrounding vegetation, which roots are associated with *T. aestivum*; and third by wild animals such as boar that could spread *T. aestivum* truffles in not fenced plantations. In summer, it is recommendable to inspect *T. melanosporum* orchards with a dog and remove *T. aestivum* truffles which are sources of spores.

We defined the *T. aestivum* contamination as the percent of *T. aestivum* kilos of truffle production yield. We established four levels of contamination according to development phases: Null, Low, High and Total (Table 3). Null means that all the production is of *T. melanosporum* truffles and Total that all is of *T. aestivum* truffles.

Table. 3 Percent of *T. aestivum* kilos of truffle production yield for development phases

Period (Year)	***T. aestivum* contamination (% *T. aestivum* kilos of truffle production yield)**			
	Null	**Low**	**High**	**Total**
0	0	0	0	0
1-5	0	0	0	0
6-14	0	5	25	100
15-30	0	10	50	100
31-40	0	15	75	100

The growth of contamination is dynamically over the years intending to evaluate the impact of *T. aestivum* without intervention.

3.1.3 Market price

Spanish Truffle Price depends weekly on global offer, mainly French demand, and quality. The maximum price is got before Christmas week and at the end of the season when truffles are in high maturation.

There are four categories of quality relative to maturity, consistency, form and size of the truffles: Extra, First, Second and Third (Table 4). Also, the price depends on the destination of the truffles: wholesale trades, retail trades or directly to restaurants or private consumers. Table 4 shows prices of T. melanosporum truffles for quality categories in two different times in a retail trade market.

T. melanosporum truffles are economically more appreciated than *T. aestivum* for its aroma and flavor. We defined three levels of price for *T. melanosporum* and *T. aestivum* truffles: Low, Medium and High (Table 5).

The Low price simulates a very unfavorable situation to know what would happen if prices drop due to a global oversupply. The Medium price means selling fresh truffles to wholesale trade and the High price to retail trade, restaurants, and private consumers.

Truffle prices can be consulted every week in the Market of Vic (http://www.llotjadevic.org/es/noticies/16/trufa), and in some local French markets (Carpentras:

http://www.carpentras.fr/pratique/foires-et-marches/marche-aux-truffes-dhiver.html; Richerenches https://rnm.franceagrimer.fr/prix?TRUFFE)

Table 4. Categories of black truffle quality and mean prices (clean truffle after soil removal) for retail trade in the 23th November 2018 and 1st February 2019

Categories	Description	Price (Nov-Feb)	Example image
EXTRA	Spherical mature truffles > 20 grams of weight. The optimum organoleptic maturation starts from the middle of December.	600€/kg (February) There is no EXTRA truffles in the beginning of the season.	 Extra truffles
1ST	Spherical truffles between 15 grams and 20 grams. Irregular truffles > 20 grams.	225-500€/kg	  Spherical truffles; Irregular truffle
2ND	Truffles less than 15 grams and big pieces of matured truffle.	125€-330€/kg	 Half truffle in organoleptic maturation
3RD	Soft, frost, and immature truffles, small pieces of truffle, and truffles with wormholes.	70-90€/kg	  Immature truffle ; small pieces of truffle
Source: Retail trade from the Lleida area			

Table. 5 Mean of *T. aestivum* and *T. melanosporum* truffle price for an average-high quality

Truffle species	Price (€/kilo)		
	Low	Medium	High
T. aestivum	50	75	100
T. melanosporum	150	350	500

3.2 Extraordinary Receivables

The only extraordinary was for the sale of holm oak firewood at the end of the project life span, in the 40th year after the truffle collection.

3.3 Result of Annual Receivables

3.3.1 Ordinary receivables

The Ordinary Annual Receivables were calculated according to defined levels of kilos of *T. aestivum* truffles, kilos of *T. melanosporum* and price per kilo of *T. aestivum* and *T. melanosporum* truffles. Table 6 shows an example for Medium truffle production yield, Low *T. aestivum* contamination and Medium price. Table 7 shows the Ordinary Annual Receivables for each development phase and defined levels of truffle production yield, *T. aestivum* contamination and price. They are in ascending order of Total Receivables for the project life span. Total Receivables were calculated as the sum of Ordinary Annual Receivables according to the development phase and the years that comprise it.

3.3.2 Extraordinary receivables

In the 40th year, there was an extraordinary receivable of 24,304.00€ for the sale of firewood. We considered a wood growth of 3.10 kg of firewood/tree and year, so for 40-years-old trees and a density of 280 trees/ha, there were 34,720 Kg of firewood/ha. The price of holm oak firewood was 0.07 euros per kilo that include logging. The firewood yield was extracted from a project of a Truffle orchard in Zaragoza. The firewood price was provided by a specialized company from the Lleida area.

Table. 6 Example of Ordinary Annual Receivables for Medium truffle production yield, Low contamination and Medium price

Year	Truffle yield	Kg truffles/ha and year	*T. aestivum* contamination	% Kg *T. aest* of truffle yield	Kg *T. aest*/ha	Kg *T. mel* /ha	Price	€/Kg *T. aest*	€/Kg *T. mel*	€/ha	Total 10 ha
0	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
1	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
2	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
3	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
4	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
5	Medium	0	Low	0	0.00	0.00	Medium	75	350	0.00	0.00 €
6	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
7	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
8	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
9	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
10	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
11	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
12	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
13	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
14	Medium	10	Low	5	0.50	9.50	Medium	75	350	3,362.50	33,625.50 €
15	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
16	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
17	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
18	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
19	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
20	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
21	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
22	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €

Year	Truffle yield	Kg truffles/ha and year	*T. aestivum* contamination	% Kg *T. aest* of truffle yield	Kg *T. aest*/ha	Kg *T. mel* /ha	Price	€/Kg *T. aest*	€/Kg *T. mel*	€/ha	Total 10 ha
23	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
24	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
25	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
26	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
27	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
28	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
29	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
30	Medium	30	Low	10	3.00	27.00	Medium	75	350	9,675.00	96,750.50 €
31	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
32	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
33	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
34	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
35	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
36	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
37	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
38	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
39	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €
40	Medium	15	Low	15	2.25	12.75	Medium	75	350	4,631.25	46,312.50 €

Table 7. Ordinary Annual Receivables for each development phase, in ascending order of Total Receivables

Truffle yield	*T. aestivum* contamination	Price	0-5	6-14	15-30	31-40	Total
Low	Total	Low	0,00 €	2.500,00 €	7.500,00 €	3.750,00 €	180.000,00 €
Low	Total	Medium	0,00 €	3.750,00 €	11.250,00 €	5.625,00 €	270.000,00 €
Low	High	Low	0,00 €	6.250,00 €	15.000,00 €	5.625,00 €	352.500,00 €
Medium	Total	Low	0,00 €	5.000,00 €	15.000,00 €	7.500,00 €	360.000,00 €
Low	Total	High	0,00 €	5.000,00 €	15.000,00 €	7.500,00 €	360.000,00 €
Low	Low	Low	0,00 €	7.250,00 €	21.000,00 €	10.125,00 €	502.500,00 €
Medium	Total	Medium	0,00 €	7.500,00 €	22.500,00 €	11.250,00 €	540.000,00 €
Low	Null	Low	0,00 €	7.500,00 €	22.500,00 €	11.250,00 €	540.000,00 €
Medium	High	Low	0,00 €	12.500,00 €	30.000,00 €	11.250,00 €	705.000,00 €
Medium	Total	High	0,00 €	10.000,00 €	30.000,00 €	15.000,00 €	720.000,00 €
High	Total	Low	0,00 €	10.000,00 €	30.000,00 €	15.000,00 €	720.000,00 €
Low	High	Medium	0,00 €	14.062,50 €	31.875,00 €	10.781,25 €	744.375,00 €
Medium	Low	Low	0,00 €	14.500,00 €	42.000,00 €	20.250,00 €	1.005.000,00 €
Low	High	High	0,00 €	20.000,00 €	45.000,00 €	15.000,00 €	1.050.000,00 €
Medium	Null	Low	0,00 €	15.000,00 €	45.000,00 €	22.500,00 €	1.080.000,00 €
High	Total	Medium	0,00 €	15.000,00 €	45.000,00 €	22.500,00 €	1.080.000,00 €
Low	Low	Medium	0,00 €	16.812,50 €	48.375,00 €	23.156,25 €	1.156.875,00 €
Low	Null	Medium	0,00 €	17.500,00 €	52.500,00 €	26.250,00 €	1.260.000,00 €
High	High	Low	0,00 €	25.000,00 €	60.000,00 €	22.500,00 €	1.410.000,00 €
High	Total	High	0,00 €	20.000,00 €	60.000,00 €	30.000,00 €	1.440.000,00 €
Medium	High	Medium	0,00 €	28.125,00 €	63.750,00 €	21.562,50 €	1.488.750,00 €
Low	Low	High	0,00 €	24.000,00 €	69.000,00 €	33.000,00 €	1.650.000,00 €
Low	Null	High	0,00 €	25.000,00 €	75.000,00 €	37.500,00 €	1.800.000,00 €
High	Low	Low	0,00 €	29.000,00 €	84.000,00 €	40.500,00 €	2.010.000,00 €
Medium	High	High	0,00 €	40.000,00 €	90.000,00 €	30.000,00 €	2.100.000,00 €
High	Null	Low	0,00 €	30.000,00 €	90.000,00 €	45.000,00 €	2.160.000,00 €
Medium	Low	Medium	0,00 €	33.625,00 €	96.750,00 €	46.312,50 €	2.313.750,00 €
Medium	Null	Medium	0,00 €	35.000,00 €	105.000,00 €	52.500,00 €	2.520.000,00 €
High	High	Medium	0,00 €	56.250,00 €	127.500,00 €	43.125,00 €	2.977.500,00 €
Medium	Low	High	0,00 €	48.000,00 €	138.000,00 €	66.000,00 €	3.300.000,00 €
Medium	Null	High	0,00 €	50.000,00 €	150.000,00 €	75.000,00 €	3.600.000,00 €
High	High	High	0,00 €	80.000,00 €	180.000,00 €	60.000,00 €	4.200.000,00 €
High	Low	Medium	0,00 €	67.250,00 €	193.500,00 €	92.625,00 €	4.627.500,00 €
High	Null	Medium	0,00 €	70.000,00 €	210.000,00 €	105.000,00 €	5.040.000,00 €
High	Low	High	0,00 €	96.000,00 €	276.000,00 €	132.000,00 €	6.600.000,00 €
High	Null	High	0,00 €	100.000,00 €	300.000,00 €	150.000,00 €	7.200.000,00 €

3.3.3 *T. aestivum* Contamination on Total Receivables

In a Medium price, Total Receivables decreased 8.18%, 40.92%, 78.57% of Null contamination Receivables for Low, High and Total contamination respectively (Table 8). The Percentages were the same for Low, Medium and High truffle yield levels because the levels were defined proportionally.

Table 8. Total Receivables and percentages of its losses for a Medium price, levels of *T. aestivum* contamination, and truffle yield production

Truffle yield	***T. aestivum* Contamination**			
	Null	**Low**	**High**	**Total**
Low	1.260.000,00 €	1.156.875,00 €	744.375,00 €	270.000,00 €
Medium	2.520.000,00 €	2.313.750,00 €	1.488.750,00 €	540.000,00 €
High	5.040.000,00 €	4.627.500,00 €	2.977.500,00 €	1.080.000,00 €
(In relation to Null)	100%	-8.18%	-40.92%	-78.57%

In a Medium truffle production yield and Total contamination, Total Receivables decreased 66.87%, 78.57% and 80.00% of Null contamination Receivables for Low, Medium and High price, respectively (Table 9). As price increases, there is more money not earned due to *T. aestivum* truffles and their lower price than of *T. melanosporum*. Percentages were calculated as in Table 7 but for the price levels, instead of doing for the truffle yield levels.

Table 9. Percentages of Total Receivables losses for *T. aestivum* contamination and price levels

Price	***T. aestivum* Contamination**			
	Null	**Low**	**High**	**Total**
Low	100%	-6.94%	-34.72%	-66.67%
Medium	100%	-8.18%	-40.92%	-78.57%
High	100%	-8.33%	-41.67%	-80.00%

4. ANNUAL PAYABLES

In a truffle orchard, Ordinary Payables are the payments of the activities that every season or frequently we need to do for producing and collecting truffles. In contrast, Extraordinary Payables are those difficult to predict and not frequent such as material renewal. We considered that all the activities were contracted to an external company or were done by temporary workers. All the payments were estimated as if the owner of the *T. melanosporum* orchard has not been participating in the activities.

The Annual Payables were defined independently of Truffle yield production because we cannot predict if we will have more or less production at the beginning of the season. The time to collect truffles with dogs was considered the same.

4.1 Ordinary Payables

In general, the labors and associated payments needed to produce and collect truffles could be: Irrigation, Soil aeration and weed removal, Pruning and Truffle collection.

4.1.1 Irrigation

In the proposed example, there is no payment for water, but there is for the necessary electricity to raise the water from the water well to the irrigation pond. Also, we included the hours of irrigation programming and maintenance. We distinguish the irrigation after plantation, the support irrigation, and the production irrigation.

4.1.2 Weed removal and soil aeration

We distinguish two types of weed removal and soil aeration: one done by a tractor between trees and one manually around seedlings. By tractor it was scheduled in March, May, July and September from plantation to the 5th year and once every season in April when the plantation starts to produce. Manually was done after the tractor tilling from plantation to the 8 year.

4.1.3 Pruning

There were two types of pruning: first and shape pruning in the year 3 and 5, and annual pruning for the phases of entry into production, productive potential and stability and decrease of production.

4.1.4 Truffle collection

The total of hours for collect *T. melanosporum* and *T. aestivum* in contamination conditions is the same that the total for collect just *T. melanosporum* truffles without contamination. The truffle production yield is the same for all the contamination levels. It only changes the amount of *T. melanosporum* and *T. aestivum* truffles. For management purposes, all truffle collection took place between the months of the *T. melanosporum* season although *T. aestivum* collection takes place during the summer.

It also includes the annual payments associated with dogs and care, such as feeding, vaccines, and other veterinary expenses.

4.2 Extraordinary Payables

We identified as extraordinary Payables the purchase of trained dogs to collect truffles and the partial renewal of irrigation system and other materials.

4.2.1 Purchase of trained dog for truffle collection

The first purchase of dogs was considered as an investment in 6th year. It was a total payment of 4,537.50€ to buy five dogs. The following purchases of dogs during the project life span were defined as extraordinary payables did it in the years 13, 20, 27 and 34. We considered seven years as the mean of work life of a trained dog for collecting truffles.

4.2.2 Partial renewal of irrigation system and other renewals

In the 21st year, there was a partial renewal of the irrigation system. It was considered a payment of the 35% of investment on irrigation, including materials and installation. In the same year we considered another renewal to change some materials. It was defined as 25% of the investment of the irrigation pond, water irrigation hut, and fence material and installation.

4.3 Result of Annual Payables

4.3.1 Ordinary Annual Payables

The Ordinary Annual Payables are summarized in the Table 10 and decomposed by activities in the Table 11, 12, 13 and 14, for the year 0, to 1 to 5 year, to 6 to 14, and to 15 to 40 year, respectively.

Table 10. Summarized Ordinary Annual Payables for each year or period

Year or period	Development phases	Annual Payables
0	Plantation	8,992.48€
1, 2, 4	Unproductive	8,727.73€
3, 5	Unproductive	9,018.13€
6, 7, 8	Entry into production	23,582.90€
9-14	Entry into production	22,977.90€
15-40	Productive potential and stability, and decrease of production	27,817.90€

Table 11. Ordinary Annual Payables decomposed by activities and payments for the Plantation phase (Year 0)

Time	Activity	Activity description	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
October	Support irrigation after planting	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10 ha					96.80 €
March	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
March	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
April	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10 ha					96.80 €
May	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10 ha					96.80 €
May	Surface soil labor	Weed removal and soil aeration: Tractor driver +150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
May	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
June	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
July	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
August	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
September	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
Once	Electricity payment for plantation and support irrigation	Irrigation after planting and support. 97.18€+IVA/ha + 1500€+IVA/year. Each irrigation is about 10L/m^2. Support irrigation is 2-4 times/month.	Direct payment for 10ha					2,990.88 €
							Total Ordinary Annual Payables for the year 0	**8,992.48 €**

Table 12. Ordinary Annual Payables decomposed by activities and payments for the Unproductive phase (Period to 1 to 5 year)

Time	Activity	Activity description	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
March	Surface soil labor	Weed removal and soil aeration: Tractor driver +150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
March	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
April	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
May	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
May	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
May	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
June	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
July	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
August	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Support irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
September	Soil seedling labor	Manual weed removal around seedlings	Hours	5	10.00	50.00	60.50	605.00 €
Once	Electricity payment for support irrigation	83.30€+IVA/ha + 1500€+IVA/year. Each irrigation is about 10L/m^2 and 2-4 times/month.	Direct payment for 10ha					2,822.93 €
							Total Ordinary Annual Payables for the years 1, 2 and 4	**8,727.73 €**
Time	**Activity**	**Activity description**	**Factor**	**Units/ha**	**€/unit**	**€/ha**	**€/ha+VAT**	**Total: 10ha**
(Year 3 and 5) February	Young pruning	Laborer for first and shape pruning	Hours	3	8.00	24.00	29.04	290.40 €
							Total Ordinary Annual Payables for the years 3 and 5	**9,018.13 €**

Table 13. Ordinary Annual Payables decomposed by activities and payments for the Entry into production phase (Period to 6 to 14 year)

Time	Activity	Activity description	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
Once	Truffle collection	Laborer for truffle collection. November-May	Daily wage	5	80.00	400.00	484.00	4,840.00 €
March	Production pruning	Annual pruning	Hours	32	10.5	336.00	406.56	4,065.60 €
April	Surface soil labor	Weed removing and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
April	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
May	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
June	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
August	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
Once	Electricity payment for production irrigation	655€+IVA/ha + 1500€+IVA/year. Each irrigation is about 20-30L/m^2 and 2-4 times/month.	Direct payment for 10ha					9,740.50 €
Once	Dog feed and care	Dog feed and care. 500€+IVA/dog	Direct payment for 10ha					3,025.00 €
							Total Ordinary Annual Payables for the period to 9 to 14 years	**22,977.90 €**
Time	**Activity**	**Activity description**	**Factor**	**Units/ha**	**€/unit**	**€/ha**	**€/ha+VAT**	**Total 10ha**
(Year 6, 7 and 8) April	Soil seedling labor	Manual weed remove around seedlings	Hours	5	10	50	60,50	605,00 €
							Total Ordinary Annual Payables for the period to 6 to 8 years	**23,582.90 €**

Table 14. Ordinary Annual Payables decomposed by activities and payments for the Productive potential and stability phase, and Decrease of production period phase (Period to 15 to 40 year)

Time	Activity	Activity description	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
Once	Truffle collection	Laborer for truffle collection. November-May	Daily wage	10	80.00	800.00	968.00	9,680.00 €
March	Production pruning	Annual pruning	Daily wage	32	10.50	336.00	406.56	4,065.60 €
April	Surface soil labor	Weed removal and soil aeration: Tractor driver+150CV Tractor+plough	Hours	1	60.00	60.00	72.60	726.00 €
April	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
May	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
June	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
July	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
August	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
September	Production irrigation	Laborer for irrigation programming and maintenance. 10hours/month and 8€+IVA/h	Direct payment for 10ha					96.80 €
Once	Electricity payment for production irrigation	655€+IVA/ha + 1500€+IVA/year. Each irrigation is about 20-30L/m^2 and 2-4 times/month.	Direct payment for 10ha					9,740.50 €
Once	Dog feed and care	Dog feed and care. 500€+IVA/dog	Direct payment for 10ha					3,025.00 €
			Total Ordinary Annual Payables for the period to 15 to 40 years					**27,817.90 €**

4.3.2 Extraordinary Annual Payables

There are Extraordinary Annual Payables in the year 13, 20, 21, 27 and 34 (Table 15).

Table 15 Extraordinary Annual Payables by decomposed activities

Time	Activity	Activity description	Factor	Units/ha	€/unit	€/ha	€/ha+VAT	Total 10ha
13, 20, 27, 34 years **Before November**	Purchase of truffle dog	Trained dog for truffle collection. 1 dog every 2ha. 750+IVA/dog	Direct payment for 10ha					4,537.50 €
Year 21 September	Partial Renewal	Partial Renewal of irrigation system. 35% of investment on irrigation, including materials and installation.	Direct payment per hectare			1,351.00€	1,634.71 €	16,347.10 €
Year 21 September	Other renewals	Change of materials and other renewals. 25% of the construction of the irrigation pond, irrigation hut and fence materials and installation.	Direct payment for 10ha					13,444.61 €
Total Ordinary Annual Payables for the years 13, 20, 27 and 34								**4,537.50€**
Total Ordinary Annual Payables for the year 21								**29,791.71 €**

5. CASH FLOW BETWEEN RECEIVABLES AND PAYABLES

The Ordinary Cash Flow is the difference between Ordinary Annual Receivables (O. Receivables) and Ordinary Annual Payables (O. Payables). It depends on Truffle production yield, contamination and price levels. The Extraordinary Cash Flow is the difference between Extraordinary Annual Receivables (E. Receivables) and Extraordinary Annual Payables (E. Payables). The Annual Receivables and Payables were calculated in the previous sections, *3. ANNUAL RECEIVABLES* and *4. ANNUAL PAYABLES*.

5.1 Result of cash flow

Table 16 presents an example of Ordinary and Extraordinary cash flow for each year and for a Medium Truffle yield production, Null contamination and Medium price. Table 17 for a Medium truffle yield production, High contamination and Medium price. The Ordinary Cash Flow for High contamination is negative during plantation, unproductive and the decrease of production phase when for Null contamination is not for the decrease of the production phase.

Table 16. Cash flow for Medium truffle production yield, Null contamination and Medium price

Year	O. Receivables	O. Payables	Ordinary Cash Flow	E. Receivables	E. Payables	Extraordinary Cash Flow
0	0.00 €	8,992.48 €	-8,992.48 €	0.00 €	0.00 €	0.00 €
1	0.00 €	8,727.73 €	-8,727.73 €	0.00 €	0.00 €	0.00 €
2	0.00 €	8,727.73 €	-8,727.73 €	0.00 €	0.00 €	0.00 €
3	0.00 €	9,018.13 €	-9,018.13 €	0.00 €	0.00 €	0.00 €
4	0.00 €	8,727.73 €	-8,727.73 €	0.00 €	0.00 €	0.00 €
5	0.00 €	9,018.13 €	-9,018.13 €	0.00 €	0.00 €	0.00 €
6	35,000.00 €	23,582.90 €	11,417.10 €	0.00 €	0.00 €	0.00 €
7	35,000.00 €	23,582.90 €	11,417.10 €	0.00 €	0.00 €	0.00 €
8	35,000.00 €	23,582.90 €	11,417.10 €	0.00 €	0.00 €	0.00 €
9	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	0.00 €	0.00 €
10	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	0.00 €	0.00 €
11	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	0.00 €	0.00 €
12	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	0.00 €	0.00 €
13	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	4,537.50 €	-4,537.50 €
14	35,000.00 €	22,977.90 €	12,022.10 €	0.00 €	0.00 €	0.00 €
15	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
16	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
17	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
18	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
19	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
20	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	4,537.50 €	-4,537.50 €
21	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	29,791.71 €	-29,791.71 €
22	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
23	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
24	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
25	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
26	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
27	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	4,537.50 €	-4,537.50 €
28	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
29	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
30	105,000.00 €	27,817.90 €	77,182.10 €	0.00 €	0.00 €	0.00 €
31	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
32	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
33	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
34	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	4,537.50 €	-4,537.50 €
35	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
36	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
37	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
38	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
39	52,500.00€	27,817.90 €	24,682.10 €	0.00 €	0.00 €	0.00 €
40	52,500.00€	27,817.90 €	24,682.10 €	24,304.00 €	0.00 €	24,304.00 €

In yellow, variable money that depends on truffle production yield, contamination and price levels

Table 17. Cash flow for Medium truffle production yield, Null contamination and Medium price

Year	O. Receivables	O. Payables	Ordinary Cash Flow	E. Receivables	E. Payables	Extraordinary Cash Flow
0	0,00 €	8,992.48 €	-8.992,48 €	0.00 €	0.00 €	0.00 €
1	0,00 €	8,727.73 €	-8.727,73 €	0.00 €	0.00 €	0.00 €
2	0,00 €	8,727.73 €	-8.727,73 €	0.00 €	0.00 €	0.00 €
3	0,00 €	9,018.13 €	-9.018,13 €	0.00 €	0.00 €	0.00 €
4	0,00 €	8,727.73 €	-8.727,73 €	0.00 €	0.00 €	0.00 €
5	0,00 €	9,018.13 €	-9.018,13 €	0.00 €	0.00 €	0.00 €
6	28.125,00 €	23,582.90 €	4.542,10 €	0.00 €	0.00 €	0.00 €
7	28.125,00 €	23,582.90 €	4.542,10 €	0.00 €	0.00 €	0.00 €
8	28.125,00 €	23,582.90 €	4.542,10 €	0.00 €	0.00 €	0.00 €
9	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	0.00 €	0.00 €
10	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	0.00 €	0.00 €
11	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	0.00 €	0.00 €
12	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	0.00 €	0.00 €
13	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	4,537.50 €	-4,537.50 €
14	28.125,00 €	22,977.90 €	5.147,10 €	0.00 €	0.00 €	0.00 €
15	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
16	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
17	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
18	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
19	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
20	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	4,537.50 €	-4,537.50 €
21	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	29,791.71 €	-29,791.71 €
22	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
23	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
24	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
25	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
26	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
27	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	4,537.50 €	-4,537.50 €
28	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
29	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
30	63.750,00 €	27,817.90 €	35.932,10 €	0.00 €	0.00 €	0.00 €
31	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
32	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
33	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
34	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	4,537.50 €	-4,537.50 €
35	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
36	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
37	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
38	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
39	21.562,50 €	27,817.90 €	-6.255,40 €	0.00 €	0.00 €	0.00 €
40	21.562,50 €	27,817.90 €	-6.255,40 €	24,304.00 €	0.00 €	24,304.00 €

In yellow, variable money that depends on truffle production yield, contamination and price levels

6. ECONOMIC ASSESSMENT OF THE PROJECT

6.1.1 Net Present Value (VAN)

The Net present value is the value in year 0 of all the future cash flows after discount the investments. It indicates the net profit generated by the project. When VAN>0 means that investment was recovered and it was generated capital gains. Contrary to when VAN< 0 without recovering it and without gains. When VAN=0, the investment is recovered but there are no gains.

All the cash flows were moved to year 0 by the interest (i), discount rate or the updating rate (i). Time changes de value of the money so we apply the interest to pass from the future Value to the present value using the VAN equation (Figure 1).

$$\boldsymbol{VAN = CF_0 + \frac{CF_j}{(1+i)^j} - \left(K_o + \frac{K_j}{(1+i)^j}\right)}$$
CF_0: Cash flow between all the receivables and payables from the year 0; CF_j: Cash flow from the year j
K_0: Investment in the year 0; K_j: Investment in the year j
i: interest, discount or updating rate

Figure 1. VAN equation (Romero, 1998).

The equivalent monthly VAN was presented with the aim to know if the Truffle project can be the only activity or if it is needed to combine it with other activities. We did it for different periods because the evolution of the VAN is not linear and depends on the interest and the elapsed years from the plantation, periods were: from 0 to Pay-back year, 0 to 20 year, 0 to 25 year, 0 to 30 year, 0 to 35 year, and 0 to 40 year. The equivalent monthly VAN was the result of a ratio between the VAN of the last year of the period and all the years and months into the period as in the example (Expression 1). It means that the VAN is equivalent to X monthly VAN during a period of 21 years.

$$X\ monthly\ VAN\ (0-20\ year) = \frac{X\ VAN\ in\ the\ 20\ year}{21\ years}\ x\ \frac{1\ year}{12\ months}$$

Expression 1. Equivalent monthly VAN for a period of years

It will be considered that the Truffle project can be the only activity when the equivalent VAN is equal or more than 1500€/month in most of the periods. It can be the main activity when it is between 750-1500€/month and be a support activity when it is 300-750€/month. We considered that in an equivalent VAN <300€/month is not worth investing in the project.

6.1.2 Payback

It is the time elapsed to recover the investment and start to generate capital gains. So shorter paybacks mean more attractive investments.

6.1.3 Benefit/Investment (Q)

It is the ratio between Benefit and Investment (Figure 2). It is a relative expression of profitability and means the gains obtained for each euro invested in the project. There are gains when Q is over 1.

$$\boldsymbol{Q = \frac{VAN}{K_o + \frac{K_j}{(1+i)^j}}}$$

Figure 2. Q equation (Romero, 1998).

6.1.4 Discount rate (TIR)

The TIR expresses the opportunity cost of choosing one alternative and forgoing another. It is a relative expression of profitability and is calculated when VAN=0 (Figure 3).

$$\boldsymbol{VAN = CF_0 + \frac{CF_j}{(1+TIR)^j} - \left(K_o + \frac{K_j}{(1+TIR)^j}\right) = 0}$$

Figure 3. TIR equation (Romero, 1998).

When TIR>i it means that the project give more profitability than other activity or bank interest. Contrary to when TIR<i where the bank offers a better alternative than the project. When TIR= I, it means that leave the money in the bank produces the same profitability as investing it in the project. We defined a 4% interest. It is a protected interest because it is high, and it will take more time to recover the investment than with a lower. Also, our project needs to get an interest over 4% to be the best alternative.

6.2 Result of VAN Analysis

Figure 4 shows the applied equations of VAN, Q and TIR for the project, with a project life span of 40 years, an interest of 4% and two investments, one in the 0 year and one in the 6 year.

Equation of VAN
$$\boldsymbol{VAN = CF_0 + \frac{CF_1}{(1+0.04)^1} + \cdots + \frac{CF_{40}}{(1+0.04)^{40}} - \left(K_o + \frac{K_6}{(1+0.04)^6}\right) = Summary_0 + \frac{Summary_1}{(1+0.04)^1} + \cdots + \frac{Summary_{40}}{(1+0.04)^{40}}}$$ $(VAN\ by\ Excel) \rightarrow \boldsymbol{Summary_0 + VNA\ function\ (4\%; Summary_1: Summary_{40}}$
Equation of Q
$$\boldsymbol{Q = \frac{VAN}{K_o + \frac{K_6}{(1+0.04)^6}}}$$
Equation of TIR
$$\boldsymbol{VAN = CF_0 + \frac{CF_1}{(1+TIR)^1} + \cdots + \frac{CF_{40}}{(1+TIR)^{40}} - \left(K_o + \frac{K_6}{(1+TIR)^6}\right) = 0 = Summary_0 + \frac{Summary_1}{(1+TIR)^1} + \cdots + \frac{Summary_{40}}{(1+TIR)^{40}}}$$ $(TIR\ by\ Excel) \rightarrow \boldsymbol{TIR\ function\ (Summary_0: Summary_{40})}$
CF: Cash flow between annual receivables and payables
Summary: Cash flow between investments, receivables and payables.

Figure 4. Applied economic equations for the project.

Table 18 shows an example of results of VAN evolution and economic parameters for Medium truffle yield production, Null contamination and Medium price. In red are negative cash flows, the investments and negative VAN evolution while the investments are not recovered. The results for the rest of the levels were obtained in the same way.

Table 18. VAN result for Medium truffle yield production, Null contamination and Medium price

Year	Investment	O. Cash Flow	E. Cash Flow	Summary	VA	Evolution VAN
0	-220,809.06 €	-8,992.48 €	0.00 €	-229,801.54 €	-229,801.54 €	-229,801.54 €
1		-8,727.73 €	0.00 €	-8,727.73 €	-8,392.05 €	-238,193.59 €
2		-8,727.73 €	0.00 €	-8,727.73 €	-8,069.28 €	-246,262.87 €
3		-9,018.13 €	0.00 €	-9,018.13 €	-8,017.08 €	-254,279.95 €
4		-8,727.73 €	0.00 €	-8,727.73 €	-7,460.50 €	-261,740.45 €
5		-9,018.13 €	0.00 €	-9,018.13 €	-7,412.25 €	-269,152.70 €
6	-45,677.50 €	11,417.10 €	0.00 €	-34,260.40 €	-27,076.49 €	-296,229.19 €
7		11,417.10 €	0.00 €	11,417.10 €	8,676.06 €	-287,553.13 €
8		11,417.10 €	0.00 €	11,417.10 €	8,342.36 €	-279,210.77 €
9		12,022.10 €	0.00 €	12,022.10 €	8,446.57 €	-270,764.20 €
10		12,022.10 €	0.00 €	12,022.10 €	8,121.70 €	-262,642.50 €
11		12,022.10 €	0.00 €	12,022.10 €	7,809.33 €	-254,833.17 €
12		12,022.10 €	0.00 €	12,022.10 €	7,508.97 €	-247,324.20 €
13		12,022.10 €	-4,537.50 €	7,484.60 €	4,495.06 €	-242,829.15 €
14		12,022.10 €	0.00 €	12,022.10 €	6,942.46 €	-235,886.68 €
15		77,182.10 €	0.00 €	77,182.10 €	42,856.48 €	-193,030.20 €
16		77,182.10 €	0.00 €	77,182.10 €	41,208.15 €	-151,822.05 €
17		77,182.10 €	0.00 €	77,182.10 €	39,623.23 €	-112,198.82 €
18		77,182.10 €	0.00 €	77,182.10 €	38,099.25 €	-74,099.57 €
19		77,182.10 €	0.00 €	77,182.10 €	36,633.90 €	-37,465.67 €
20		77,182.10 €	-4,537.50 €	72,644.60 €	33,154.05 €	-4,311.62 €
21		77,182.10 €	-29,791.71 €	47,390.39 €	20,796.49 €	16,484.87 €
22		77,182.10 €	0.00 €	77,182.10 €	32,567.40 €	49,052.27 €
23		77,182.10 €	0.00 €	77,182.10 €	31,314.81 €	80,367.08 €
24		77,182.10 €	0.00 €	77,182.10 €	30,110.39 €	110,477.48 €
25		77,182.10 €	0.00 €	77,182.10 €	28,952.30 €	139,429.78 €
26		77,182.10 €	0.00 €	77,182.10 €	27,838.75 €	167,268.53 €
27		77,182.10 €	-4,537.50 €	72,644.60 €	25,194.35 €	192,462.89 €
28		77,182.10 €	0.00 €	77,182.10 €	25,738.49 €	218,201.38 €
29		77,182.10 €	0.00 €	77,182.10 €	24,748.55 €	242,949.93 €
30		77,182.10 €	0.00 €	77,182.10 €	23,796.68 €	266,746.61 €
31		24,682.10 €	0.00 €	24,682.10 €	7,317.26 €	274,063.87 €
32		24,682.10 €	0.00 €	24,682.10 €	7,035.83 €	281,099.70 €
33		24,682.10 €	0.00 €	24,682.10 €	6,765.22 €	287,864.92 €
34		24,682.10 €	-4,537.50 €	20,144.60 €	5,309.15 €	293,174.07 €
35		24,682.10 €	0.00 €	24,682.10 €	6,254.83 €	299,428.90 €
36		24,682.10 €	0.00 €	24,682.10 €	6,014.26 €	305,443.15 €
37		24,682.10 €	0.00 €	24,682.10 €	5,782.94 €	311,226.09 €
38		24,682.10 €	0.00 €	24,682.10 €	5,560.52 €	316,786.61 €
39		24,682.10 €	0.00 €	24,682.10 €	5,346.65 €	322,133.26 €
40		24,682.10 €	24,304.00 €	48,986.10 €	10,203.27 €	332,336.53 €
			VAN=	331,336.53 €		Interest = 4%
			Q=	1.29 €		
			Payback	21 years		
			TIR=	7.89%		

6.2.1 Results without *T. aestivum* contamination

High truffle production yield

In a High truffle production yield, the investment was recovered in all the levels of price (Figure 5).

VAN=	2,285,062.54 €	VAN=	1,384,056.52 €	VAN=	182,090.81 €
Q=	8.89 €	Q=	5.39 €	Q=	0.71 €
Payback	11 years	Payback	15 years	Payback	24 years
TIR=	18.33%	TIR=	14.67%	TIR=	6.40%
Price: High		Price: Medium		Price: Low	

Figure 5. Result of economic parameters for High truffle production yield, Null contamination and price levels.

The TIR was higher than the interest of the bank in all the price levels (>4%), so the *T. melanosporum* orchard is the best alternative. Although the Truffle project was always the best alternative in this analysis, the VAN in Low price was equivalent to a low monthly benefit of 32.21 € for 25 years, 105.73€ for 26 years, 363.59€ for 31 years, 364.92€ for 35 years or 370.10€ for 41 years (Table 19). So the Truffle project in Low price cannot be the only activity but can be a support activity. Contrary to in Medium and High prices that can be the only activity, with a monthly equivalent benefit over 1,500€ in most of the periods.

Table 19. Equivalent monthly VAN for periods and truffle price levels for High truffle production yield and Null contamination

	Truffle price levels		
Period	**High**	**Medium**	**Low**
0-Payback year	(0-11 year): 175.20€	(0-15 year): 412.33 €	(0-24 year): 32.21 €
0-20 year	3,901.70 €	2,093.02 €	-
0-25 year	4,881.93€	2,834.99 €	105.73 €
0-30 year	5,312.15€	3,191.34 €	363.59 €
0-35 year	4,959.79€	2,990.56 €	364.92 €
0-40 year	4,645.39€	2,813.12 €	370.10 €

In conclusion, without *T. aestivum* contamination, the investment of the project is protected by High truffle yield production in the assumption that a global oversupply drops *T. melanosporum* price down to 150€/kg. The High truffle production yield with High Price was the maximum potential of the project. It must be considered that in high production conditions is difficult to sell all the truffles at high prices and will be necessary to develop some investments in marketing to achieve it, not contemplated in this analysis.

Medium truffle production yield

In a Medium truffle production yield the investment was not recovered in Low price but it was in High and Medium prices (Figure 6). In these conditions. The TIR was higher than the interest of the bank (>4%) so they were benefits.

VAN=	783,073.67 €	VAN=	332,336.53 €	VAN=	-268,646.32 €
Q=	3.05 €	Q=	1.29 €	Q=	-1.05 €
Pay-back	17 years	Pay-back	21 years	Pay-back	Not recovered
TIR=	11.39 %	TIR=	7.89%	TIR=	-3.68%
Price: High		Price: Medium		Price: Low	

Figure 6. Result of economic parameters for Medium truffle production yield, Null contamination and price levels.

The Truffle project could be the only activity in High price with an equivalent monthly VAN > 1,500€ on most of the periods, and a support activity in Medium price with an equivalent monthly VAN >300€ (Table 20). In High and Medium prices there was a high relative profitability Q, for each euro invested was earned more than one euro, and the maximum payback took place not too far from the half of project life span.

Table 20. Equivalent monthly VAN for periods and truffle price levels for Medium truffle production yield and Null contamination

	Truffle price levels	
Period	**High**	**Medium**
0-Payback year	(0-17 year): 238.82€	(0-21 year): 62.44 €
0-20 year	887.23 €	553.29 €
0-25 year	1,470.36€	446.89 €
0-30 year	1,777.47€	717.06 €
0-35 year	1,677.74€	693.12 €
0-40 year	1,591.61€	675.48 €

In conclusion, without *T. aestivum contamination*, the investment of the project was recovered when the sale of the *T. melanosporum* truffles was at prices ≥350€/kg for a Medium truffle production yield. In these conditions, the project had a high relative profitability.

The Low price in Medium truffle field conditions won't be analyzed in contamination levels given that the project was not feasible without contamination.

Low truffle production yield

In a Low truffle production yield the investment was only recovered in High price (Figure 7). For this price level the TIR was higher than the interest of the bank (>4%). Even though the investment was recovered, it was in a high payback, after 30 years. Also, the equivalent monthly VAN was less than 300€/month (Table 21). So we consider that it is not worth investing in the project.

VAN=	31,845.10 €	VAN=	-203,604.05 €	VAN=	-494,014.89 €
Q=	0.12 €	Q=	-0.79 €	Q=	-1.92 €
Pay-back	30 years	Pay-back	Not recovered	Pay-back	Not recovered
TIR=	4,48%	TIR=	-1.05%	TIR=	-
Price: High		Price: Medium		Price: Low	

Figure 7. Result of economic parameters for Low truffle production yield, Null contamination and price levels.

Table 21. Equivalent monthly VAN for periods and Low truffle production yield, Null contamination and High price

Period	High price
0-Payback year	(0-30 year): 10.12€
0-35 year	36.71€
0-40 year	64.73€

In conclusion, although there were no *T. aestivum* contamination, the benefits of Low truffle production yield were not enough for being a support activity, for the High, Medium and Low price levels defined. The mean price would have to be >500€/kg to improve the profitability of the project. It is unlikely to get those prices. So the Truffle project has not enough in annual productions ≤5 kg/ha in 6-14 years, ≤15Kg/ha in 15-30 years, and ≤7.5 Kg/ha in 31-40 years.

The Low truffle yield production won't be analyzed in contamination levels given that the project was not feasible without contamination.

6.2.2 Results with *T. aestivum* contamination

High price

In a High truffle production yield, the investment was not recovered in Total contamination due to collect just *T. aestivum* truffles instead of *T. melanosporum* (Figure 8). The VAN decreased 10.09% and 50.51% of Null contamination for Low and High, contamination respectively. The payback in High contamination took place two years later than in Null contamination. There was a loss of 0.89€ for each euro invested in the project for Low contamination and of 4.49€ for High contamination in comparison to Null contamination.

VAN=	2,285,062.54 €	VAN=	2,054,578.41 €
Q=	8.89 €	Q=	8.00 €
Payback	11 years	Pay-back	11 years
TIR=	18.33%	TIR=	17.69%
Contamination: Null		Contamination: Low	
VAN=	1,130,768.89 €	VAN=	-118.400,61 €
Q=	4.40 €	Q=	-0.46 €
Pay-back	13 years	Pay-back	Not recovered
TIR=	14,49%	TIR=	2%
Contamination: High		Contamination: Total	

Figure 8. Result of economic parameters for High truffle production yield, High price and contamination levels.

The Truffle project could be the only activity in Low and High contamination as in Null contamination, according to their equivalent monthly VAN (Table 22).

Table 22. Equivalent monthly VAN for periods and High truffle production yield, contamination levels, and High price

	***T. aestivum* contamination levels**		
Period	**Null**	**Low**	**High**
0-Payback year	(0-11 year): 175.20€	(0-11 year): 55.52 €	(0-13 year): 36.86 €
0-20 year	3,901.70 €	3,516.39 €	1,975.15 €
0-25 year	4,881.93€	4,414.43 €	2,544.42 €
0-30 year	5,312.15€	4,812.31 €	2,812.97 €
0-35 year	4,959.79€	4,812.31 €	2,521.76 €
0-40 year	4,645.39€	4,175.97 €	2,298.31 €

In conclusion, the Total contamination produces that the investment of the project is not recovered in High truffle yield production. In conditions, without *T. aestivum* the investment was always recovered. The high production of *T. aestivum* truffles was not profitable for the annual payables defined, but it doesn't mean that the *T. aestivum* orchards are never profitable. Although *T. aestivum* price per kilo is lower than of *T. melanosporum* its orchards have: lower necessities of water (most of them don't have an irrigation system) than *T. melanosporum*; and higher production yield. So the analysis of the economic profitability of *T. aestivum* orchards would be according to their specific payables and receivables. In Low and High contamination, the Truffle Project still has high relative profitability: TIR>14%, Q >4€ and the payback <20 years. Also, at these levels, the project could continue being the only activity. So except in Total contamination, the investment and profitability of the project are protected by annual productions ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, and mean prices ≥ 500€/kg for *T. melanosporum* truffles and ≥ 100€/kg for *T. aestivum* truffles.

In a Medium truffle production yield, the investment was not recovered in Total contamination (Figure 9). The VAN decreased 15.18% and 73.73% of Null contamination for Low and High contamination, respectively. The payback in High contamination took place five years later than in Null contamination. There was a loss of 0.45€ for each euro invested in the project for Low contamination and of 2.25€ for High contamination in comparison to Null contamination. The Truffle project could be the main activity in Low contamination and a support activity in High contamination, according to their equivalent monthly VAN (Table 23).

VAN=	783,073.67 €	VAN=	667,597.48 €
Q=	3.05 €	Q=	2.60 €
Pay-back	17 years	Pay-back	17 years
TIR=	11.39 %	TIR=	10.72%
Contamination: Null		Contamination: Low	
VAN=	205,692.71 €	VAN=	-418.892,04 €
Q=	0.80 €	Q=	-1.63 €
Pay-back	22 years	Pay-back	Not recovered
TIR=	7,03%	TIR=	-
Contamination: High		Contamination: Total	

Figure 9. Result of economic parameters for Medium truffle production yield, High price and contamination levels.

Table 23. Equivalent monthly VAN for periods and Medium truffle production yield, contamination levels, and High price

	***T. aestivum* contamination levels**		
Period	**Null**	**Low**	**High**
0-Payback year	(0-17 year): 238.82€	(0-17 year): 93.20 €	(0-22 year): 77.13 €
0-20 year	887.23 €	694.58 €	-
0-25 year	1,470.36€	1,236.61 €	301.95 €
0-30 year	1,777.47€	1,527.55 €	527.87 €
0-35 year	1,677.74€	1,433.93 €	458.72 €
0-40 year	1,591.61€	1,356.91 €	418.07 €

In conclusion, in High price and Medium truffle production yield, the investment is recovered in contaminations ≤25% of truffle production yield in 6-14 years, ≤50 in 15-30 years, and ≤75 in 31-40 years. But in contaminations ≥ 5% of truffle yield production in 6-14 years, ≥ 10% in 15-30 years, and ≥ 15% in 31-40 years the Truffle project could not be the only activity to develop.

Medium price

In a High truffle production yield, the investment was only not recovered in Total contamination (Figure 10). The VAN decreased 11.47% and 57.36% of Null contamination for Low and High contamination, respectively. In comparison to Null contamination, there was a loss of 0.62€, and 3.09€ for each euro invested in the project for Low and High contamination, respectively.

VAN=	1,384,056.52 €	VAN=	1,225,276.76 €
Q=	5.39 €	Q=	4.77 €
Pay-back	15 years	Pay-back	15 years
TIR=	14.67%	TIR=	14.04%
Contamination: Null		Contamination: Low	
VAN=	590,157.71 €	VAN=	-268,646.32 €
Q=	2.30 €	Q=	-1.05 €
Pay-back	16 years	Pay-back	No recovered
TIR=	10,77%	TIR=	-4.00%
Contamination: High		Contamination: Total	

Figure 10. Result of economic parameters for Medium truffle production yield, Medium price and contamination levels.

The Truffle project could be the only activity in Low contamination and the main activity in High contamination, according to their equivalent monthly VAN (Table 24).

Table 24. Equivalent monthly VAN for periods and High truffle production yield, contamination levels, and Medium price

	T. aestivum **contamination levels**		
Period	**Null**	**Low**	**High**
0-Payback year	(0-15 year): 412.33 €	(0-15 year): 277.08 €	(0-16 year): 12.50 €
0-20 year	2,093.02 €	1,828.12 €	768.51 €
0-25 year	2,834.99 €	2,513.58 €	1,227.95 €
0-30 year	3,191.34 €	2,847.70 €	1,473.15 €
0-35 year	2,990.56 €	2,691.88 €	1,314.41 €
0-40 year	2,813.12 €	2,490.40 €	1,199.51 €

In conclusion, exempt the Total contamination, the recovering of the investment in contamination levels is protected by annual productions ≥20 kg/ha in 6-14 years, ≥60Kg/ha in 15-30 years, and ≥30 Kg/ha in 31-40 years, and mean prices ≥350€/kg for *T. melanosporum* truffles and ≥ 75€/kg for *T. aestivum* truffles. The Truffle project in Low and High contamination can still be the only activity.

In a Medium truffle production yield, the investment was not recovered in High and Total contamination (Figure 11). The VAN decreased 23.92%, of Null contamination for Low contamination. The payback in Low contamination took place one year later than in Null contamination. There was a loss of 0.31€ for each euro invested in the project in comparison to Null contamination.

VAN=	331,868.27 €	VAN=	252,478.39 €
Q=	1.29 €	Q=	0.98 €
Pay-back	21 years	Pay-back	22 years
TIR=	7,89%	TIR=	7.21%
Contamination: Null		Contamination: Low	
VAN=	-65,081.14 €	VAN=	-494.483,15 €
Q=	-0.25 €	Q=	-1.92 €
Pay-back	Not recovered	Pay-back	Not recovered
TIR=	3,00%	TIR=	-2.80%
Contamination: High		Contamination: Total	

Figure 11. Result of economic parameters for Medium truffle production yield, Medium price and contamination levels.

In Low contamination, the equivalent monthly VAN was over 300 € in most of the periods so the Truffle project could be a support activity combined with others (Table 25).

Table 25. Equivalent monthly VAN for periods and Medium truffle production yield, contamination levels, and Medium price.

	T. aestivum **contamination levels**	
Period	**Null**	**Low**
0-Payback year	(0-21 year): 62.44 €	(0-22 year): 31.06 €
0-20 year	553.29 €	-
0-25 year	446.89 €	286.19 €
0-30 year	717.06 €	545.24 €
0-35 year	693.12 €	525.51 €
0-40 year	675.48 €	514.12 €

In conclusion, in Medium truffle production and Medium price, the investment is not recovered in contaminations ≥ 25% of truffle yield production in 6-14 years, ≥ 50% in 15-30 years, and ≥ 75% in 31-40 years. But the Truffle project continues being a support activity as without contamination.

Low price

In a High truffle production yield, the investment was not recovered in High and Total contamination (Figure 12). The VAN decreased 31.70%, of Null contamination for Low contamination. The payback in Low contamination takes place two years later than in Null contamination, and there was a loss of 0.23€ for each euro invested in the project in comparison to Null contamination.

VAN=	182,090.81 €	VAN=	124,352.72 €
Q=	0.71 €	Q=	0.48 €
Pay-back	24 years	Pay-back	26 years
TIR=	6.40%	TIR=	6.00%
Contamination: Null		Contamination: Low	
VAN=	-106,599.66 €	VAN=	-418.892,04 €
Q=	-0.41 €	Q=	-1.63 €
Pay-back	Not recovered	Pay-back	Not recovered
TIR=	2,00%	TIR=	-
Contamination: High		Contamination: Total	

Figure 12. Result of economic parameters for High truffle production yield, Low price and contamination levels.

The Truffle project could be a support activity combined with others in Null contamination. In Low contamination, it is not worth investing in the project because of its low equivalent monthly VAN (Table 26).

Table 26. Equivalent monthly VAN for periods and High truffle production yield, contamination levels, and Low price.

	***T. aestivum* contamination levels**	
Period	**Null**	**Low**
0-Payback year	(0-24 year): 32.21 €	(0-26 year): 51.82 €
0-20 year	-	-
0-25 year	105.73 €	-
0-30 year	363.59 €	238.63 €
0-35 year	364.92 €	243.02 €
0-40 year	370.10 €	252.75 €

In conclusion, in contamination levels ≥ 5% of truffle production yield in 6-14 years, ≥ 10% in 15-30 years, and ≥ 15% in 31-40 years, the benefits of High production yield were not enough for being a support activity in Low price.

7. CONCLUSIONS

A truffle production ≤ 5 kg/ha in 6-14 years, ≤ 15Kg/ha in 15-30 years, and ≤ 7.5 Kg/ha in 31-40 years is not profitable for the defined cash flow and project investment.

<u>Without *T. aestivum* contaminations, the activity is profitable in these conditions</u>:

- *T. melanosporum* production is ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, and mean price is ≥ 150€/kg.
- *T. melanosporum* production is ≥ 10 kg/ha in 6-14 years, ≥ 30Kg/ha in 15-30 years, and ≥ 15 Kg/ha in 31-40 years, and mean price is ≥ 350€/kg.

But it could be the only activity to develop in these conditions:

- *T. melanosporum* production is ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, and mean price is ≥ 350€/kg.
- *T. melanosporum* production is ≥ 10 kg/ha in 6-14 years, ≥ 30Kg/ha in 15-30 years, and ≥ 15 Kg/ha in 31-40 years, and mean price is ≥ 500€/kg.

<u>With *T. aestivum* contaminations the activity is profitable in these conditions</u>:

- Truffle production is ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, contaminations are ≤ 5% of truffle production yield in 6-14 years, ≤ 10% in 15-30 years, and ≤ 15% in 31-40 years, and mean price is ≥ 150€/kg for *T. melanosporum* and ≥ 50€/kg for *T. aestivum*.
- Truffle production is ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, contaminations are ≤ 25% of truffle production yield in 6-14 years, ≤ 50% in 15-30 years, and ≤ 75% in 31-40 years, and mean price is ≥ 350€/kg for *T. melanosporum* and ≥ 75€/kg for *T. aestivum*.
- Truffle production is ≥ 10 kg/ha in 6-14 years, ≥ 30Kg/ha in 15-30 years, and ≥ 15 Kg/ha in 31-40 years,, contaminations are ≤ 5% of truffle production yield in 6-14 years, ≤ 10% in 15-30 years, and ≤ 15% in 31-40 years, and mean price is ≥ 350€/kg for *T. melanosporum* and ≥ 75€/kg for *T. aestivum*.
- Truffle production is ≥ 10 kg/ha in 6-14 years, ≥ 30Kg/ha in 15-30 years, and ≥ 15 Kg/ha in 31-40 years, contaminations are ≤ 25% of truffle production yield in 6-14 years, ≤ 50 in 15-30 years, and ≤ 75 in 31-40 years, and mean price is ≥ 500€/kg for *T. melanosporum* and 100€/Kg for *T. aestivum*.

But it could be the only activity to develop in these conditions:

- Truffle production is ≥ 20 kg/ha in 6-14 years, ≥ 60Kg/ha in 15-30 years, and ≥ 30 Kg/ha in 31-40 years, contaminations are ≤5% of truffle yield production in 6-14 years, ≤ 10% in 15-30 years, and ≤ 15% in 31-40 years, and mean price is ≥ 350€/kg for *T. melanosporum* and ≥ 75€/kg for *T. aestivum*.

- Truffle production is ≥ 10 kg/ha in 6-14 years, ≥ 30Kg/ha in 15-30 years, and ≥ 15 Kg/ha in 31-40 years, contaminations are when contamination are ≤ 5% of truffle yield production in 6-14 years, ≤ 10% in 15-30 years, and ≤ 15% in 31-40 years, and mean price is ≥ 500€/kg for *T. melanosporum* and 100€/kg for *T. aestivum*.

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